CHAPTER 1

INTRODUCTION

The chicken (*Gallus gallus domesticus*) has three main chemoreceptive systems, olfaction, gustation and the common chemical sense (Kare and Mason, 1986). The sense of smell detects volatile cues by means of a receptor sheet located in a caudal chamber of the nasal cavity, whereas the sense of taste detects non-volatile stimuli in the oral cavity. The neural system responsible for the common chemical sense consists predominantly of the free nerve endings of the trigeminal nerve, which are located in the mucous membranes of the nasal cavity, eyes and oral cavity, as well as the outer surface of the beak. This system responds to many volatile chemicals.

Some of the earliest studies examining avian olfaction, most notably by Audubon (1826) and Darwin (1834), concluded that odours are unlikely to play a role in the behaviour of birds. Reports that birds possess a sense of smell were still inconclusive at the turn of the century (Strong, 1911; Gurney, 1922). However, the long-held notion that birds are completely anosmic is not consistent with the anatomical, electrophysiological and behavioural evidence of a well-developed olfactory sense (Cobb, 1960; Stager, 1967; Bang, 1971; Bang and Wenzel, 1985; Wenzel, 1987). Avian olfaction has been examined mainly in domesticated species, such as the chick and the pigeon. While the neuroanatomy of the olfactory system has been described for the pigeon (for example, Rieke and Wenzel, 1975; 1978; Macader *et al.*, 1980; Reiner and Karten, 1985), the gross anatomy of the avian nasal cavity has been described for the chick (Bang, 1961; 1971). In addition, the chick is used frequently as a model species for the embryological development of the olfactory system (Ayer-Le Lièvre *et al.*, 1995).

ONSET OF OLFACTORY FUNCTION IN THE CHICK

By day 20 of incubation (day E20), the chick embryo is responsive to a number of different odorants (Tolhurst and Vince, 1976). It is not known whether the chick embryo responds to odorants before day E20 but the onset of olfactory responsiveness may be on day E20, as this is when the chick begins to breathe air. The embryo is surrounded by amniotic fluid until the beak pierces the surrounding membranes late on day E19. Day E20 marks a dramatic change for the chick embryo as it begins to breathe with its lungs (Vince, 1976) and this must have important effects on the embryo's ability to respond to odorants in the air sac. Furthermore, the external nares are covered with tissue for the greater part of incubation (Romanoff, 1960) but on day E18 the translucent peridermal covering of the beak begins to peel off allowing air to pass through the nasal cavities (Hamburger and Hamilton, 1951). The Bowman's glands, situated within the olfactory epithelium, begin secretory activity by day E19 (Mendoza, 1980) and this aqueous medium is required for the adsorption of odorous molecules. It is, however, possible that the olfactory system is stimulated before day E19 by chemicals found in the amniotic fluid, particularly as the chick embryo begins to swallow the surrounding amniotic fluid from day E8 (Kuo, 1932). This would depend on whether the swallowed amniotic fluid reaches the nasal cavity of the chick and this is not known. To do so, the swallowed fluid would have to enter the nasal cavity through a retro-nasal route.

Tolhurst and Vince (1976) removed the portion of the shell surrounding the air sac of day E20-21 embryos and presented various odours, including 1-2 dichloroethane, cineole, amyl acetate and formic acid, which elicited a number of physiological and behavioural responses. To measure heart rate electrodes were attached to the outer portion of the shell while the behaviour of the chick was scored by direct observation. The behaviours measured included beak clapping, head shaking, gapes, vocalisations, small head movements, wriggles and vibrations. Using this procedure it was possible to assess unlearnt responses to various concentrations of each of the odours. The responses to the odours used were likely to have been caused by the odour acting on receptors within the nasal cavity because they were abolished when the nostrils of the

embryo were occluded with wax. However, Tolhurst and Vince (1976) were unable to determine whether the responses were mediated by the olfactory receptors or the free nerve endings of the trigeminal nerve that line the nasal cavity, even though there are structural and functional differences between these two systems.

Head shaking responses to odorants by the embryo and the post-hatching chick

Tolhurst and Vince (1976) reported that the most reliable responses to odours included increases in heart rate, beak clapping and head shaking. Tolhurst and Vince concluded that head shaking was the most useful index of responsiveness to odours because it was observed primarily following the presentation of odour. By contrast, beak clapping was thought, by Tolhurst and Vince, to be an inaccurate indicator of olfactory responsiveness as it is a prevalent aspect of embryonic activity.

Odours also evoke head shaking when presented together with (Vallortigara and Andrew, 1994) or without (Guilford *et al.*, 1987) conspicuous visual cues during the first few days post-hatching. Therefore head shaking would seem to be a useful measure of olfactory responsiveness in the newly hatched chick as well as the embryo. However, head shaking is also evoked by visual (Andrew 1975b), gustatory (Cherkin, 1969), tactile (Hogan, 1965) and auditory (Kruijt, 1964) stimuli and, thus, it may also be evoked in potentially fearful or aversive situations.

Head shaking involves a rapid sideways movement of the head. Kruijt (1964) studied the Burmese red jungle fowl (*Gallus gallus spadiceus*) and he observed similar patterns of head shaking in domestic and wild strains of chicken. Kruijt described head shaking as a relevant movement if it occurred during feeding, used to pulverise food to edible size or to remove material from the bill or head, or during preening. According to Kruijt, irrelevant head shaking movements are observed in conflict situations, such as when a meal worm is presented, or in situations where aggression and escape responses occur simultaneously. Hogan (1965), Archer (1974), Duncan and Wood-Gush (1972) and Andrew (1974) have shown that head shaking is readily evoked by the presentation of a conspicuous visual stimulus. Furthermore, as bouts of head shaking are frequently

observed following a period of fixating a visual stimulus, head shaking has also been referred to as a transitional behaviour (Andrew, 1975b).

The reliability of head shaking as a measure of olfactory responsiveness would, however, depend largely on the frequency with which it occurs following the presentation of odorant. For example, Hogan (1965) observed 75 bouts of head shaking by chicks in 432 min (0.17 bouts min^{-1}) before and after presentation of a meal worm, whereas while the meal worm was present the frequency of head shaking increased to 124 bouts in 432 min (0.29 bouts min^{-1}). By contrast, Tolhurst and Vince (1976) reported that presenting an odorant to the chick embryo resulted in an increase of 1-5 bouts of head shaking during a 30 s trial (2-10 bouts min^{-1}). Thus, the increased frequency of head shaking bouts displayed by chicks following the presentation of odorant indicates that it is a reliable measure of olfactory responsiveness.

RESPONSES TO ODORANTS BY THE POST-HATCHING CHICK

Chicks are useful for examining responses to odours as they appear to detect volatile substances primarily by the olfactory system. Although the ophthalmic division of the trigeminal nerve is sensitive to stimulation by odours, birds do not possess an accessory olfactory system (Bang, 1971) nor do they possess a terminal nerve after hatching (Meyer *et al.*, 1987), whereas these chemoreceptive systems are present in mammalian species, such as rodents (Bojsen-Møller, 1975). Furthermore, the chick, which is a precocial species, develops rapidly after hatching and displays a range of complex behavioural patterns independently of parental assistance. This allows the newly hatched chick to be tested in a wide range of easily controlled, behavioural tests.

Although the olfactory system of the chick is functional at least one day before hatching, it is not known whether the chick can form memories of odorants presented before hatching. However, as discussed below, the chick does learn about odours soon after hatching and the chick's memory of an odour is important for forming attachments (such as imprinting on siblings or to the hen), avoidance of predators and/or learning to feed, including learning to avoid ingestion of harmful substances.

The majority of experiments examining the responses of chicks to odour have involved testing during the first week post-hatching using an approach-avoidance paradigm (i.e. Jones and Black, 1979; Jones and Gentle, 1985; Vallortigara and Andrew, 1994). These tests involve placing a chick in a test box, a Y-shaped maze or a runway and recording the amount of time the chick spends near or in an odour-treated section of the test apparatus. These behavioural studies have been complemented by physiological studies, such as stimulation of the olfactory nerve (Tucker, 1965). Both of these approaches have shown that chickens detect and respond to odours.

Measuring behavioural responses to presentation of an odour in a freely moving animal means that visual, auditory and tactile cues, as well as those from stimulation by odour, need to be considered. By restraining a pigeon in a sound and light attenuated chamber and then delivering odorous stimuli, Walker *et al.* (1986) attempted to test solely for responses to odour. However, this procedure has many limitations. For example, the bird is able to regulate its breathing pattern and, as neural activity within the olfactory bulb is synchronised with inspiration and the rate of nasal air flow (Tucker, 1965), the bird can alter the amount of odour that reaches the olfactory epithelium. Tucker (1965) was able to overcome this problem by presenting odour directly to the olfactory nerve and recording changes in electrical activity. Using this technique he found that there was a concentration-dependent increase in electrical activity in the olfactory nerve of adult chickens with increasing concentrations of odour. This provides clear evidence that the olfactory nerve is functional in the chicken but gives no indication of how an odour affects the chicken s behaviour.

Studies examining olfactory function in the chick during the first week post-hatching have shown that behaviour can be modulated by odours. Jones and Black (1979) observed responses of 7-day-old chicks placed in a cage containing conspecific blood, red or blue dye, or water. The chicks spent more time freezing, sitting and lying, and less time spent within 75 mm of the dish containing blood than the other stimuli. It is likely that chicks respond to the odour of blood, and not to its colour, as they showed

more fearful reactions when presented with blood in an open dish compared to blood contained in a sealed dish in which the blood was still visible (Jones and Black, 1979).

The effects of odorants on feeding behaviour

Olfaction is thought to contribute to the arousal and food recognition stage in food ingestion by birds (Kuenzel, 1983; Gentle, 1985). Jones (1987a) found that 8-day-old chicks show neophobic reactions, including a longer latency to commence feeding and shorter duration of feeding bouts, following the presentation of food that has been scented with a novel odour, such as orange oil. It may be that chicks respond to a novel odorant as aversive.

Chicks can also learn to associate an odour with an unpleasant feeding experience. Turro *et al.* (1994) conducted two experiments to look at the effects of odours on the feeding behaviour of domestic chicks at 2-3 days of age. In a first experiment, chicks housed in groups of 4 were exposed for 10 min to two feeders that had been scented with orange oil. Six drops of orange oil were applied to a piece of filter paper and placed under a 2 cm deep layer of starter mash in each feeder. The feeders were removed and half of the chicks received an intraperitoneal injection of lithium chloride (LiCl) and half were treated with saline. After 4 h of food deprivation, the chicks were given a choice between an orange-scented feeder and an untreated feeder in a test that lasted for 10 min. They found that LiCl-treated chicks spent less time feeding, had a lower pecking frequency and consumed less food from the orange-oil scented feeder compared to chicks treated with saline.

In a second experiment, chicks were given a choice test between an orange-scented feeder and one scented with geraniol. In this experiment, chicks had previously been exposed to either orange-scented or geraniol-scented feeders and then half were treated with LiCl and half with saline. They found that LiCl-treated chicks spent less time feeding, had a lower pecking frequency and consumed less food from the familiar scented feeder than they did from a feeder containing the novel odour. By contrast, the feeding behaviour of saline-treated chicks was the same from both of the scented-feeders (Turro

et al., 1994). Thus, chicks form an association between odour and an aversive experience.

The effects of odorants on the development of attachments

Chicks demonstrate preferences for a familiar odour that has not been paired with an aversive experience. For example, Jones and Faure (1982) and Jones and Gentle (1985) found that chicks prefer familiar wood-litter compared to clean wood-litter or wood-litter over which a strange chick has been reared. This was tested by placing the chick in the centre of a Y-maze and recording the time the chick spent in the separate arms of the maze. Since the chicks were reared and tested on wire mesh over wood litter the odours from their excreta may be served as familiar cues.

Jones and Gentle (1985) demonstrated that chicks show a preference for a reagent grade odour by rearing them with the odour of orange or geranium oil throughout the first week of life post-hatching. The chicks were tested at 7 or 8 days of age in a wooden home box with odour at one end and water at the other end. They demonstrated a preference for the odour-treated section compared to the water-treated section. Furthermore, this preference was due to the formation of a memory for the odour because the chicks did not show a preference for orange or geranium oil when tested on day 1 post-hatching. This was shown by placing the day-old chicks in a home box with a dish containing odour-treated litter (either orange or geranium oil) at one end and water treated litter at the other (Jones and Gentle, 1985). Similar to the study by Tolhurst and Vince (1976), these authors demonstrated that the responses were mediated by receptors within the nasal cavity because 7-day-old chicks tested with both nostrils occluded with dental acrylic did not demonstrate a preference for a familiar odour (geranium oil).

Experimental evidence supports the hypothesis that chicks form memories of specific odours, as they are able to discriminate between a familiar and an unfamiliar odour (Jones and Gentle, 1985). Chicks reared over litter treated with geranium oil and tested in a home box with geranium oil at one end and orange oil at the other end spend

longer in the section containing the geranium-scented litter. When the familiar odour is present the chicks are also less fearful in an otherwise novel situation (an open field; Jones and Gentle, 1985).

Recall of specific odours has also been examined using tests that are commonly used for recall of a visual imprinting stimulus. Chicks reared individually with cylinders containing an odour suspended in the home-cage can be tested for recall of that odour in a laneway with cylinders containing the familiar odour at one end and an unscented cylinder at the other end (Vallortigara and Andrew, 1994). Chicks that have been reared with an unscented, red cylinder for the first 3 days of life post-hatching also demonstrate a preference for the familiar, unscented cylinder compared to a cylinder to which amyl acetate or orange oil has been added (Vallortigara and Andrew, 1994). Burne and Rogers (1995) found that chicks exposed to the odour of nesting-litter of adult chickens established a preference for this odour by day 4 post-hatching but chicks exposed to a garlic odour, a possible aversant, did not establish a preference for this odour. This differential result appears to make biological sense as the odour of nesting-litter could serve to keep the chick in the proximity of the nest during early post-hatching life, whereas an aversive odour, such as garlic, may be an inappropriate odorant for eliciting approach during early post-hatching life.

LATERALIZED RESPONSES TO ODORANTS

The responses of chicks to a familiar odour (clove oil) appear to be lateralized. Vallortigara and Andrew (1994) tested 3-day-old male chicks in a laneway with a scented cylinder at one end (5 drops of clove oil) and an unscented cylinder at the other. The chicks were tested with one of the nostrils occluded. Chicks that had been reared with a cylinder that was scented with 5 drops of clove oil and tested using their right nostril (RN; their left nostril was temporarily blocked with a malleable wax fibre mixture) preferentially approached the familiar-scented cylinder. By contrast, those chicks tested using their left nostril (LN) approached either stimulus at random.

Chicks reared with an unscented cylinder and tested in a laneway with a scented cylinder at one end (1 ml of *n*-amyl acetate, 1 ml of amyl acetate, 1 ml of orange oil or 5 drops of clove oil) and an unscented cylinder at the other also demonstrated a right nostril bias (Vallortigara and Andrew, 1994). That is, RN chicks approached the familiar-unscented cylinder, whereas LN chicks approached both stimuli at random. Thus, depending on whether a chick has had prior exposure to an odour, RN (but not LN) chicks demonstrate approach or avoidance to a cylinder that contains odour. Therefore, a chick does not need to have formed a memory of an odour for it to show a lateralized response.

The right nostril bias for responding to the presentation of a novel odour was confirmed by Vallortigara and Andrew (1994) by presenting clove oil odour within a small peckable target. The stimulus consisted of a grey, metal box that contained a clean piece of cotton soaked with 5 drops of clove oil. The odour could diffuse out of the box through small holes situated within its walls. At test, 3-day-old male chicks, which had been reared with an unscented table tennis ball, were presented with odour in four consecutive 10 s trials with either the left or right nostril occluded. A greater percentage of RN chicks (70.6%) compared to LN chicks (27.8%) shook their heads on the first trial, indicating that they had detected the clove oil. There was also an indication that pecking was affected, although marginally, by the lateralized presentation of odour as 60-70% of RN chicks and 83% of LN chicks pecked the box on the first trial. However, LN and RN chicks responded at a similar level by the third trial.

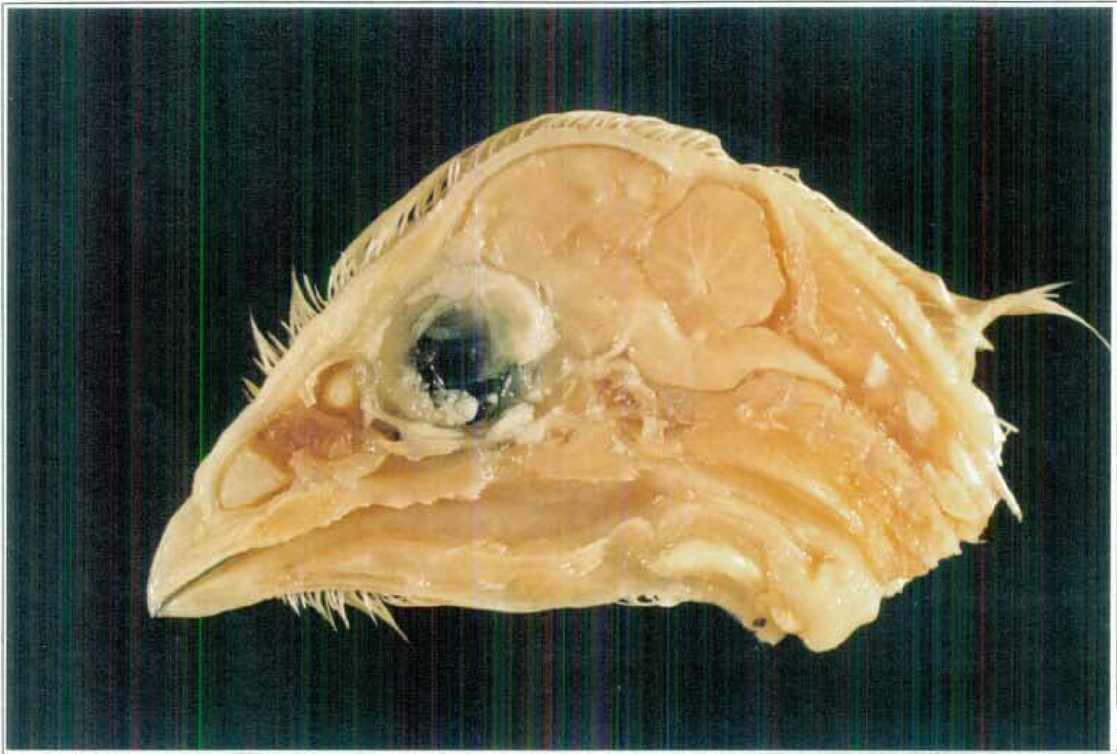
The study of Vallortigara and Andrew (1994) raises a number of issues about lateralized responses of chicks to odours but only two of them will be mentioned here. Are lateralized olfactory responses dependent on the learning paradigm in which the odorant is presented, for example, between imprinting-like odour learning and habituation learning? Furthermore, as the olfactory lateralizations were demonstrated using specific visual stimuli, either a cylinder or a box, is the lateralization caused by structural or functional asymmetries within the visual system or is the lateralization within the olfactory modality *per se*, or a combination of the two?

Lateralized responses to odours could be due to asymmetrical organisation of peripheral or central structures or right hemisphere dominance for the perception of odours. The remainder of this chapter will discuss two aspects of olfaction in the chick. First, the functioning of the chemoreceptive systems (olfactory and trigeminal) as well as the structure of these systems will be covered, including possible peripheral or central sources of asymmetry. Secondly, the interaction of olfaction with the other sensory modalities, particularly vision, will be considered.

Anatomy of the olfactory system of the chick

The peripheral, or extracranial, olfactory structures include the olfactory epithelium and the olfactory nerve (*nervus olfactorius*), whereas the central, or intracranial, olfactory structures include the olfactory bulb (*bulbus olfactorius*) and cortical and subcortical brain regions (Figure 1.1). The gross anatomy of the left and right nasal cavities (*cavitus nasalis*) of the chick is bilaterally symmetrical (Figure 1.2). The *naris* consists of hornified epithelium that continues as a flap above the nares (*operculum nasale*) and is located laterally on either side of the upper portion of the beak. The nasal cavity is divided into three areas; the nasal vestibule (*regio vestibularis*), the respiratory area (*regio respiratoria*) and the olfactory chamber (*regio olfactoria*; Bang, 1971; Bang and Wenzel, 1985). Incoming air is deflected and warmed by the vertical lamella (*lamella verticalis naris*; also known as the atrial concha [Bang, 1971]) and the rostral concha (*concha nasalis rostralis*; also referred to as the anterior [Bang, 1971] or vestibular [Croucher and Tickle, 1989] concha; see Figure 1.2.A and B). The nasal cavities are separated by a continuous cartilaginous septum (*septum nasale*; see Figures 1.2.A, B, C and D) and air entering one nostril is unable to pass directly into the opposite nasal cavity.

A



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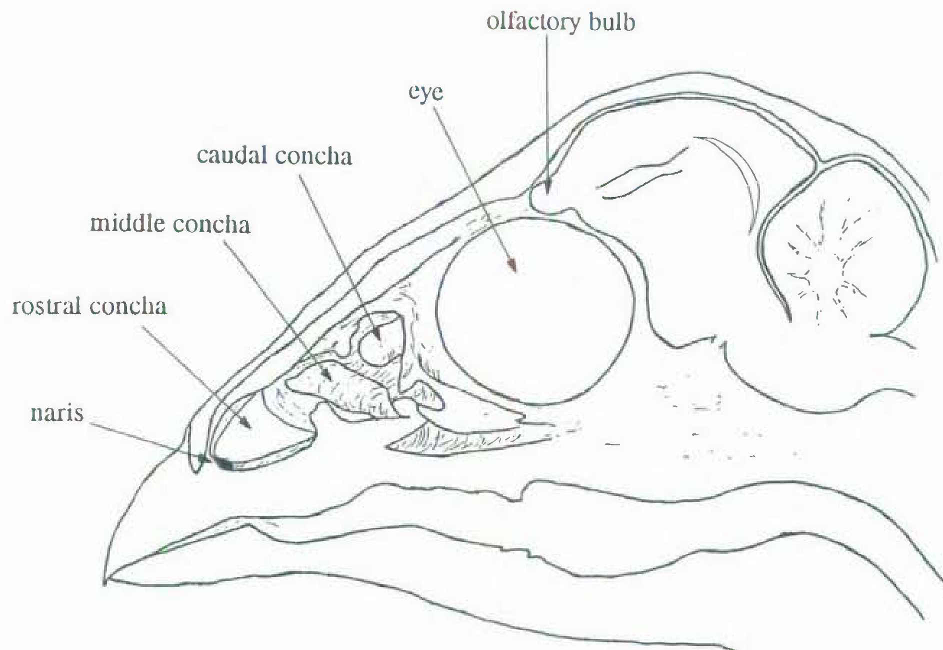

Figure 1.1 Sagittal section of the head of a 2-day old chick. A sagittal section through the midline of the head (A) and the corresponding line drawing (B) are shown to illustrate the gross anatomy of the nasal cavity.

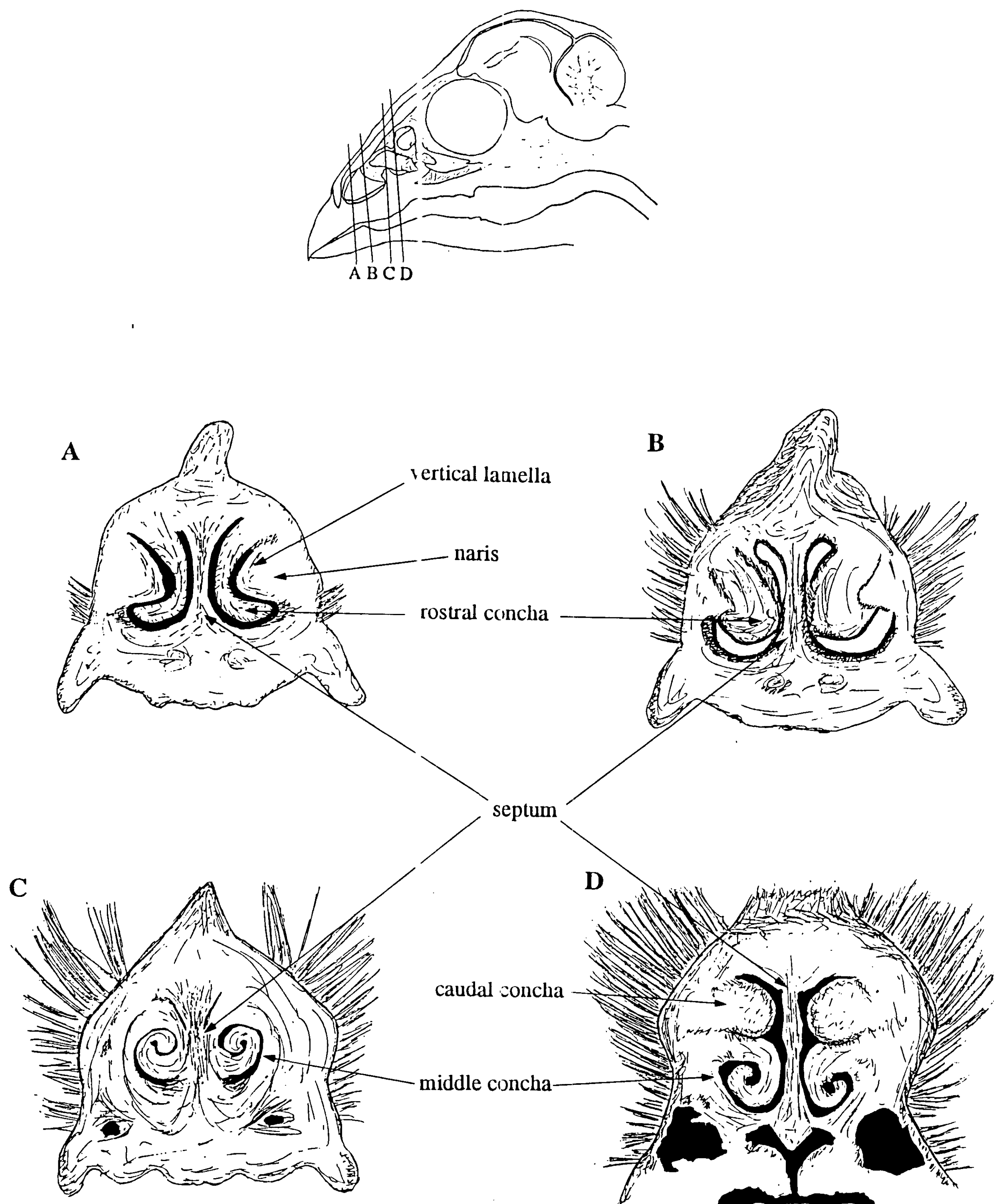

Figure 1.2 Transverse sections of the nasal cavity from a 2-day old chick. This figure shows sections taken from rostral (A, B) to caudal (C, D) regions of the nasal cavity. The key to the transverse sections is also presented in the figure. Note that the olfactory epithelium is localised on the caudal concha and adjacent septum (D). The cartilaginous septum is continuous and separates the left and right nasal cavities.

The middle concha (*concha nasalis media*; also referred to as the maxillary concha [Bang, 1971]; see Figures 1.2.C and D) further warms the inspired air and acts as a filter, trapping air particles for mucocilliary transport into the throat. Although the middle concha of birds has been referred to as the maxilloturbinal concha (Bellairs and Jenkin, 1960), the former nomenclature is preferred as it is not known whether this structure is homologous to the maxillary turbinate of mammals (King, 1979). It has been shown that the turbinal folds in the nasal cavities of humans (Principato and Ozenberger, 1970; Frye, 1995), rabbits and rodents (Bojsen-Møller and Fahrenkrug, 1971) swell and shrink at regular intervals thus reducing the flow of air through the left or right nasal cavity. This phenomenon explains the nasal cycle in which the patency of the left and right nasal cavities alternates every 2-6 h (Principato and Ozenberger, 1970).

In humans the swelling and shrinking of the left and right nasal cavities appear to cycle independently of each other (Principato and Ozenberger, 1970). This was demonstrated by a topical application of 3% ephedrine, a vasoconstrictor, into one nasal cavity, which prevented the turbinal folds from swelling. It was shown that the nasal resistance of the contralateral nasal cavity displayed the normal cycles of patency (Principato and Ozenberger, 1970). This evidence suggests that the left and right cavities function independently. Although early reports indicated that the nasal cycle was present in up to 80% of the adult human population (Heetderks, 1927, cited in Principato and Ozenberger, 1970), a more recent study (Gilbert and Rosenwasser, 1987) indicates it may occur in as few as 12% of humans and that nasal patency is episodic rather than cyclical. Nevertheless, it has been suggested that the nasal cycle causes asymmetrical changes in hemispheric processing of odours by humans (Frye, 1995) and as such may contribute to lateralized responses to odorants by the chick. However, whether this phenomenon occurs in other species is not clear.

The olfactory receptor sheet is another potential area for structural asymmetry within the olfactory system. However, olfactory neurones from the receptor families that have been characterised in the chick (nine putative receptor families termed COR1 to COR9; Nef *et al.*, 1996) are located bilaterally and symmetrically along the caudal

concha (*concha nasalis caudalis*; also referred to as the posterior [Bang, 1961] or superior [Croucher and Tickle, 1989] concha; see Figure 2D). Thus, it is unlikely that an asymmetry in the distribution or density of olfactory receptors is responsible for olfactory lateralization in the chick.

The caudal concha is innervated ventrally (*ramus ventralis*) and dorsally (*ramus dorsalis*) by branches of the olfactory nerve (Breazile and Yasuda, 1979; also referred to as internal and external branches, respectively [Watanabe and Yasuda, 1968]). Neurones project from the olfactory epithelium to form the olfactory nerve, which passes between the superior and rostral parts of the orbits and terminates in the ipsilateral olfactory bulb (Rieke and Wenzel, 1978; see Figure 1.3). The olfactory bulb is one of the most distinctively layered structures within the central nervous system (Farbman, 1992) and in the chicken consists of the glomerular, outer granule, mitral and inner granule layers (van Tienhoven and Juhasz, 1961). Much of the synaptic integration involved with olfactory processing occurs in the glomerular layer of the bulb (Farbman, 1992), the chick having about 100 glomeruli (Ayer-Le Lièvre *et al.*, 1995). There have been no reports of direct connections between the two olfactory bulbs of pigeons (Bang and Wenzel, 1985). Lesioning studies (Akutsu *et al.*, 1992), involving unilateral surgical removal of the olfactory placode on day E3.5 to 4 confirm this ipsilateral organisation, as luteinizing hormone releasing hormone immunoreactive (LHRH-ir) neurones are not found in the olfactory-forebrain axis on the side ipsilateral to the ablated placode. By contrast, the projection of LHRH-ir neurones to the telencephalon on the side contralateral to the surgery is the same as that found in unoperated embryos.

The primary projection field of the chick's olfactory system would appear to be confined to the ipsilateral hemisphere. As mentioned previously, the neural connectivity of the avian olfactory system has been established, primarily, in the domestic pigeon. Neurones originating from the mitral cell layer of the olfactory bulb form connections with areas of the forebrain hemispheres (Bang and Wenzel, 1985) and electrical stimulation of the olfactory bulb elicits two classes of responses from the ipsilateral forebrain of anaesthetised pigeons (Rieke and Wenzel, 1975; 1978). Type I responses,

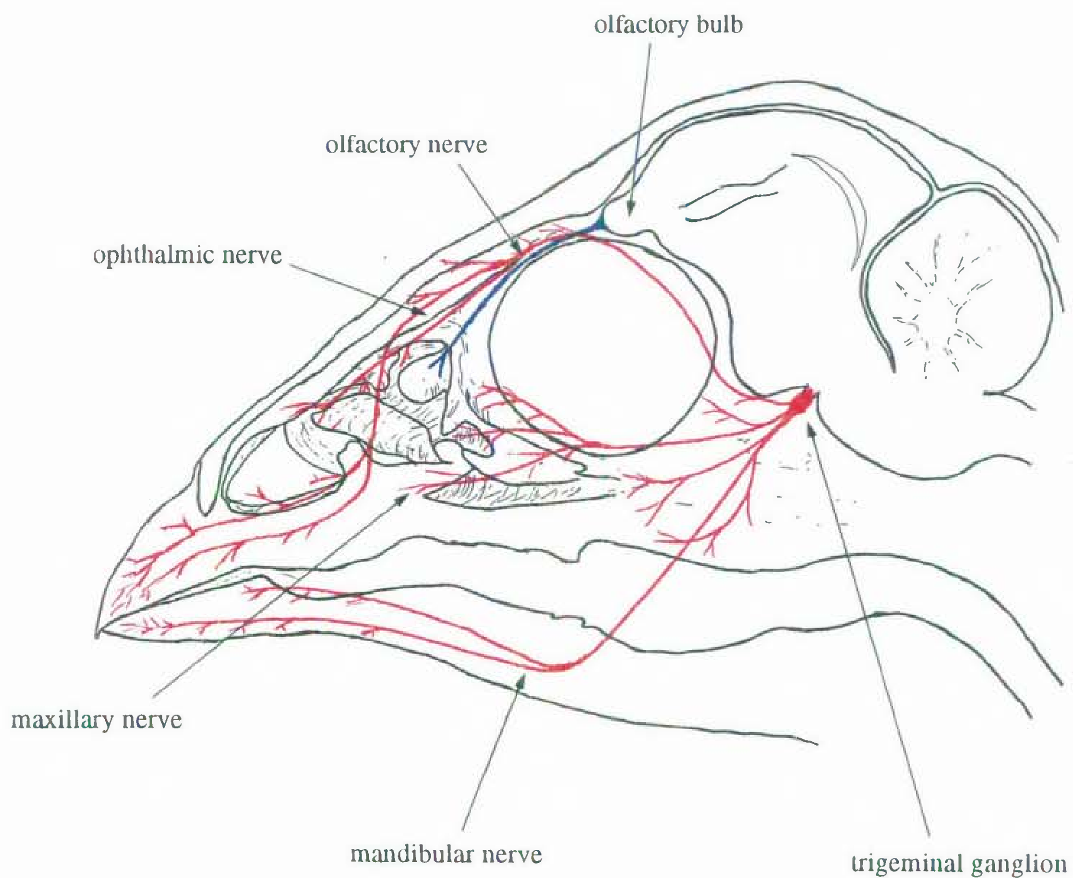

Figure 1.3 Peripheral course of the olfactory and trigeminal nerves in the chick. This figure illustrates the olfactory (blue) and trigeminal (red) nerves in a sagittal section through the midline of the head (course of nerves redrawn from Watanabe and Yasuda, 1968; Hamburger, 1961; Watanabe and Yasuda, 1970). Note that both chemoreceptive systems innervate the caudal region of the nasal cavity.

characterised by a short duration post-tetanic depression (30 ms), were recorded from areas within the primary projection field, whereas Type II responses consists of prolonged post-tetanic changes (40-90 ms) and a shorter amplitude than Type I responses. Rieke and Wenzel (1975) recorded Type I responses in the ipsilateral hyperstriatum ventrale (HV), the locus parolfactorius (LPO) and the cortex prepiriformis (CPP). The HV, LPO and CPP have been confirmed as the primary centres which receive ipsilateral connections in the pigeon by electrophysiological (Rieke and Wenzel, 1978; Macader *et al.*, 1980) and neuroanatomical studies (Rieke and Wenzel, 1978). Rieke and Wenzel (1975) recorded Type II responses, which indicate areas separated from the bulb by multiple synapses, from the hyperstriatum accessorium (HA), the hyperstriatum dorsale (HD), the nucleus septalis (S), the paleostriatum augmentatum (PA), the paleostriatum primitivum (PP) and the neostriatum (N). These authors were unable to stimulate any other areas in the ipsilateral forebrain in response to electrical stimulation of the bulb. Unfortunately, they did not measure responses in the contralateral forebrain (see Figure 1.4).

In contrast to earlier reports (Jones and Levi-Montalcini, 1958), Rieke and Wenzel (1978) found no responses in the ipsilateral archistriatum or the nucleus basalis by electrical stimulation of the bulb. Jones and Levi-Montalcini (1958) reported that fibres from the mitral cells of the bulb form the medial and lateral olfactory tracts. Axons of the medial olfactory tract (MOT) terminate in the anterior olfactory nucleus (prepiriform area), and lateral olfactory tract (LOT) end in the prepiriform area and in the ventral portion of the nucleus basalis in the paleostriatum. In addition, Reiner and Karten (1985), using tritiated proline and leucine, found labelled cells in the piriform cortex, the olfactory tubercle and the nucleus taeniae of the archistriatum (or amygdala).

Contralateral olfactory projections have been determined in pigeons by lesioning one olfactory bulb and tracing the pathway of degenerating fibres (Rieke and Wenzel, 1975; 1978). Using this technique projections were found that passed through the nucleus accumbens (Ac) of the hemisphere ipsilateral to the lesion, into the anterior commissure

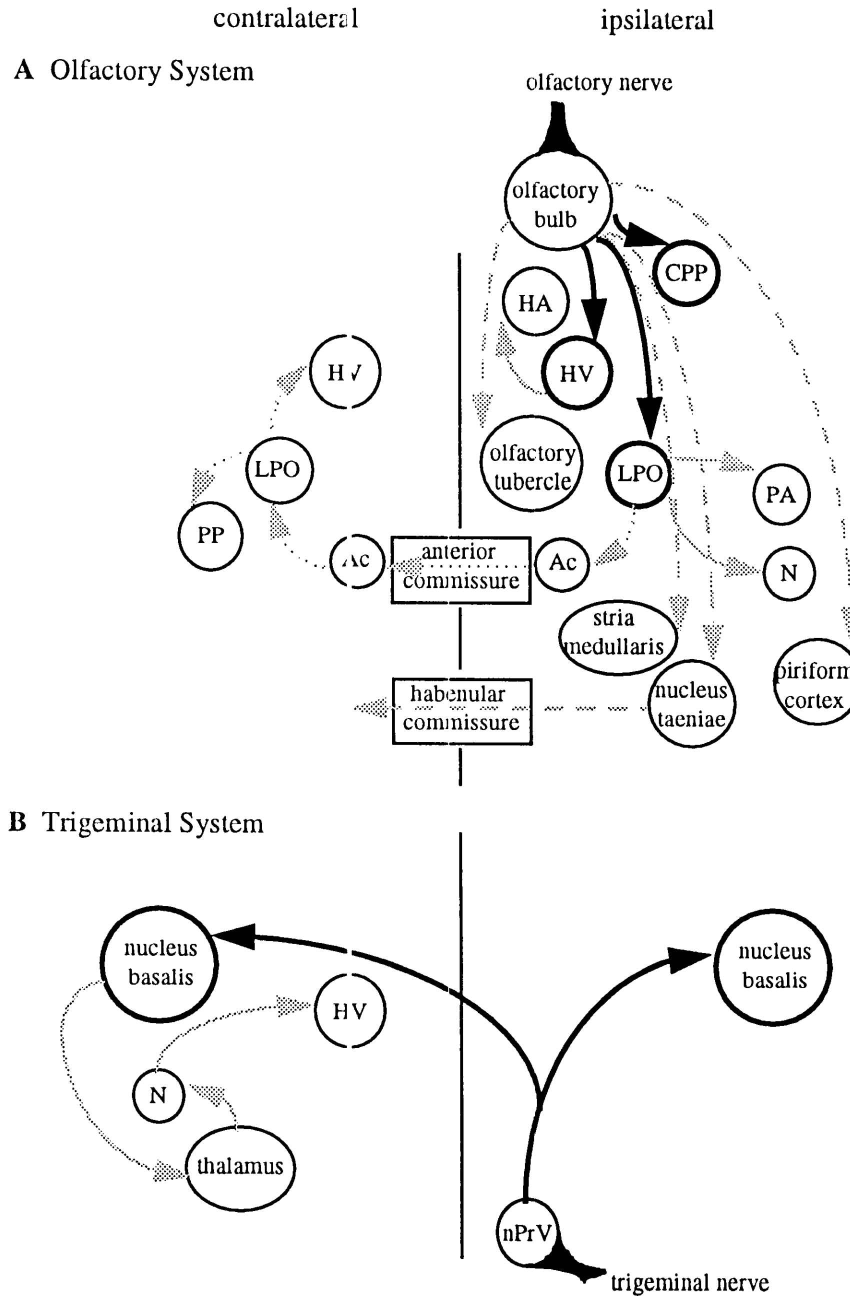

Figure 1.4 Ipsilateral and contralateral projections of the olfactory (A) and trigeminal (B) systems in the avian brain. Key to pathways (A); solid lines: electrical stimulation of olfactory nerve or olfactory bulb (Rieke and Wenzel, 1975; 1978; Macader *et al.*, 1980); dotted lines: traced pathways from lesioning one olfactory bulb (Rieke and Wenzel, 1975; 1978); dashed lines: pathways determined from autoradiographic techniques (Reiner and Karten, 1985). (B) adapted from Yasuda, 1983; Kuenzel, 1989. In (A) and (B) monosynaptic pathways from olfactory bulb or nPrV are indicated by dark lines, and polysynaptic pathways are indicated by grey lines. Note that the primary projection field of the olfactory system is to the ipsilateral hemisphere, whereas the primary projection field of the trigeminal system is to both the ipsilateral and contralateral hemispheres. Ac, nucleus accumbens, CPP, prepiriform cortex, HA, hyperstriatum accessorium, HV, hyperstriatum ventrale, LPO, lobus parolfactorius, N, neostriatum, nPrV, principal sensory trigeminal nucleus, PA, paleostriatum augmentatum, PP, paleostriatum primitivum.

and to the contralateral forebrain. These projections extend through the contralateral Ac, the PP and the caudal portion of the LPO. However, no degenerating fibres were found in the PP ipsilateral to the lesioned olfactory bulb (Rieke and Wenzel, 1978). Contralateral areas devoid of degenerating fibres included the stria medularis, habenular nuclei or the small habenular commissure, the hypothalamus, archistriatum, N or CPP. Rieke and Wenzel (1978) concluded that the LPO is the only region that has afferent fibres from both olfactory bulbs. Thus, bilateral olfactory input does occur but the interhemispheric transfer of olfactory input follows a convoluted pathway.

Gagliardo and Teyssèdre (1988) investigated the role of the anterior commissure in interhemispheric transfer of olfactory information in pigeons. They found that birds with a sectioned anterior commissure that had been habituated monolaterally to amyl acetate were still habituated to that odour when it was presented contralaterally (no indication was given by Gagliardo and Teyssèdre as to which nostril was used first or second). This implies that the pathway from the olfactory bulb via the anterior commissure to the contralateral LPO is not essential for interhemispheric transfer of olfactory information. However, using autoradiographic techniques, Reiner and Karten (1985) did not find contralateral projections from the LPO passed through the anterior commissure. Instead they found fibres from the olfactory bulb that enter the diencephalon via the stria medullaris, cross in the habenula commissure and ascend to the contralateral telencephalon. Moreover, Neuhaus (1963) reported that the tractus olfacto-habenularis ascending to the diencephalon is more highly developed than the pathways from the olfactory bulb to the forebrain.

Electrical stimulation of one olfactory nerve of pigeons, rather than stimulation of the olfactory bulb, invokes bilateral responses in the HV, including the intermediate and medial portions of the hyperstriatum ventrale (IMHV; Macader *et al.*, 1980). These authors concluded that the main ipsilateral component of the olfactory projection field in the pigeon projects monosynaptically to the HV (including the IMHV), CPP, and LPO, with secondary areas of input comprising the HA, HD, S, PA-PP, and N. Macader *et al.* (1980) concluded that in the pigeon, the potential for an interaction between sensory

modalities is present in the Wulst, or hyperstriatum, as it receives input from visual, auditory, trigeminal and olfactory fibres.

The expression and localisation of preproenkephalin (PPE) mRNA, an opioid peptide, has been compared in pigeon and chicken telencephalon (Molnar *et al.*, 1994). While similar patterns of PPE mRNA-containing cells were found in the LPO, HA, hyperstriatum intercalatum supremum (HIS), N and PA of these species, PPE mRNA-containing cells were found in the granule layer of the olfactory bulb of the pigeon but not the chicken. This was juxtaposed with PPE mRNA-containing cells extending from just dorsal of the olfactory bulb to the HD and HV in the chicken but not the pigeon (Molnar *et al.*, 1994). These differences may reflect species-specific behavioural differences. For example, the pigeon is thought to use environmental odour cues to form complex olfactory maps for homing (Waldvogel, 1989; Papi, 1990), whereas the chick does not. In the chick, the IMHV is known to play an important role in memory formation for imprinting (Horn, 1985) as well as in passive avoidance learning (Rose, 1991). Therefore, if the expression and localisation of PPE mRNA is associated with olfactory information, as suggested for the pigeon, it seems likely that in the chick the IMHV, as well as the LPO, HA, HIS, N and PA, is involved in the formation and/or consolidation of memories for odours.

Anatomy of the trigeminal system of the chick

The chick's beak and the mucous membranes of the oral and nasal cavities, as well as the eyes are innervated primarily by the trigeminal nerve (*nervus Trigeminus*; Berkhoudt *et al.*, 1982; see Figure 1.3) which is divided into three main branches. These are the ophthalmic (*nervus opthalmicus*), maxillary (*nervus maxillaris*) and mandibular (*nervus mandibularis*; Breazile and Yasuda, 1979) and they are distributed bilaterally (Hamburger, 1961). The main branch thought to be involved in the detection of odours in birds is the ophthalmic branch (Sieck and Wenzel, 1969; Walker *et al.*, 1986). The anterior portion of the nasal cavity is supplied by the ethmoid nerve (a branch of the ophthalmic nerve), whereas the posterior portion of the nasal cavity is supplied by the nasopalatine nerve (a branch of the maxillary nerve), and these are also thought to carry

chemosensory information (Silver, 1987). Curiously, the nasal cavity is also innervated by the facial (*nervus facialis*) and vagus (*nervus vagus*) nerves. Their role in chemoreception is not known.

Trigeminal chemoreception is thought to warn an animal against harmful or irritating chemical stimuli. Indeed, trigeminal responses to suprathreshold concentrations are often reflexive and protective (Farbman, 1992). However, the trigeminal system can be stimulated by non-irritating stimuli such as phenylethyl alcohol (Silver *et al.*, 1988) and trigeminal stimulation can decrease the excitability of the olfactory bulb in the absence of irritating stimuli (Stone *et al.*, 1966; 1968). Thus, there are similarities between the two chemoreceptive systems and they are clearly inter-related. However, they also differ in structure and there is evidence that the trigeminal nerves are unable to discriminate between chemical stimuli (Silver *et al.*, 1988).

The trigeminal nerve carries somatosensory information in the descending trigeminal tract (nTTD), to the principal sensory trigeminal nucleus (nPrV) and the mesencephalic nucleus (nMesV), all of which are situated in the brainstem (Berkhoudt *et al.*, 1982). The nPrV is thought to be involved in processing chemosensory information, as well as other cutaneous input from the face and beak. From the nPrV arise the quinto-frontal tract (QFT) which undergoes partial decussation and projects bilaterally and monosynaptically to the nucleus basalis (Zeigler and Karten, 1973; Dubbeldam *et al.*, 1981). There are no direct connections between the trigeminal nerve and the dorsal thalamus, an organisation which is unique to birds, and the nucleus basalis may represent a thalamic relay station (Yasuda, 1983). Delius and Bennetto (1972) have suggested that a limb of the contralateral QFT projects to the dorsal thalamus, the somatic sensory region of the neostriatum (neostriatum caudale) and, in the chick, cell bodies project to the HV (including the IMHV). Therefore, the HV may be important for the integration of information from trigeminal and olfactory tracts. It appears that, unlike rats, there is no direct trigeminal input to the olfactory bulbs of pigeons (Finger and Bottger, 1993), although this is not known for the chick.

If there was lateralization of hemispheric processing, unilateral stimulation of either nostril would involve both forebrain hemispheres by stimulation of trigeminal receptors, but stimulation of olfactory receptors would involved primarily the ipsilateral forebrain hemisphere. Lateralized responses to odorants might, therefore, be more likely to occur within the olfactory system, with its predominantly ipsilateral connections, rather than the trigeminal system, which consists of ipsilateral and contralateral fibres. However, ipsilateral connections do not, in themselves, imply lateralization, unless the hemispheres which receive these projections process that information differentially. Indeed, differential hemispheric processing has been described for the visual system and this is discussed below.

LATERALIZATION WITHIN THE VISUAL SYSTEM

Differential use of the forebrain hemispheres by the chick has been established largely in response to visual cues (Andrew and Brennan, 1985; Andrew, 1988; Andrew, 1991; Vallortigara and Andrew, 1991). Studies in which chicks have been imprinted on an object, such as a table tennis ball, or presented with a small bead and tested monocularly show specialised roles of the left and right hemispheres. It has been shown that the left hemisphere assigns stimuli to categories for rapid analysis of effective choice, whereas the right hemisphere performs detailed analysis of the stimulus, determining spatial relations and estimating novelty (Andrew, 1988; 1991).

The direction of lateralization for hemispheric processing of visual information is determined by exposure of the embryo to light during the latter part of incubation. Functional asymmetry within the visual system can be established after as little as 2 h of light exposure on day E19 (Rogers, 1982) and the normal direction of lateralization is stabilised after 2.5-6 h of asymmetrical stimulation by light on day E19 (Rogers, 1990). The asymmetry is generated because the chick embryo is positioned in the egg such that the left eye is occluded by the body, whereas the right eye is placed against the shell and can be stimulated by light that passes through the shell (Rogers, 1990). Exposing the right eye to light results in a greater number of projections from the left side of the thalamus to the hyperstriatal regions of the forebrain than from the right side of the

thalamus to the hyperstriata (Boxer and Stanford, 1985; Rogers and Sink, 1988). Furthermore, chicks incubated in darkness and then exposed to light immediately after hatching, such that both eyes are stimulated by light simultaneously, do not show structural asymmetry of visual projections from the thalamus to the hyperstriata (Rogers and Bolden, 1991).

Given that many of the tasks described thus far that have examined chicks' responses to odorants also involved presenting visual cues and that asymmetries in visual processing are determined by light the olfactory lateralization reported by Vallortigara and Andrew (1994) could, possibly, represent a form of intersensory asymmetry. That is, there may be a relationship between the direction of lateralization in response to odorants and to visual cues. The effects of visual stimulation on responses to odorants by the chick has not been investigated but it is known that exposing bobwhite quail chicks to light during incubation alters processing of auditory cues. Lickliter (1990) demonstrated that quail embryos that were incubated in darkness showed a preference for the quail maternal call, whereas those exposed to a temporally patterned light (15-W bulb pulsed at 3 cycles s^{-1}) during the latter part of incubation did not demonstrate such a preference. Moreover, quail chicks exposed to patterned light during incubation did not demonstrate a preference for a (silent) stuffed quail hen. However, these chicks demonstrated a preference for a stuffed quail hen when it was presented together with the quail maternal call (Lickliter, 1990). Thus, Lickliter and Virkar (1989) and Lickliter (1990) have shown that there is an interaction between the functioning of auditory and visual systems such that stimulation of one system (visual) alters the responses evoked by stimulation of the other system (auditory).

Although, the existence of a relationship between visual and auditory processing or visual and olfactory processing has not been examined in the domestic chick, comparison of chicks exposed to light during the latter part of incubation and those incubated in darkness may provide clues about any relationship between lateralized visual processing and lateralized responses to odorants.

OUTLINING THE AIMS OF THIS THESIS

The primary aim of this thesis was to investigate responses of 1-day-old domestic chicks to odorants. It was not known whether day-old chicks show concentration-dependent responses to odorants, or even whether they show differential sensitivity to different odorants on the day after hatching. Thus, the initial aim of the thesis was to develop a task in which odoriferous stimuli could be presented to the chick and to use this task to obtain responses from chicks presented with a number of different odorants and at various concentrations. Having performed a number of experiments to confirm the validity of the task, the aim was then to proceed to measure lateralization of responding, the effects of exposure to light during incubation on the chicks' responses to odorants and whether chicks form memories of odorants.

CHAPTER 2

GENERAL METHODS

INTRODUCTION

The procedure used for the behavioural task was the same in most of the experiments. Details of specific experiments that differed from these procedures are described in the relevant chapters.

ANIMALS

All of the experiments used white leghorn x australorp chicks. Fertile eggs, obtained from S. F. Barter and Sons, Huntingwood, NSW, were incubated and hatched at the University of New England.

The eggs were incubated for the first 17 days in a force-draft, turning incubator (Multiquip, Austral, NSW). The incubator was maintained at 37-38°C and 80% relative humidity. The eggs were held in plastic incubating trays and these were rotated through approximately 90° at least three times each day. The eggs were candled at days 8 and 17 of incubation and any non-viable embryos were removed. Fertile eggs were transferred to wire-grid hatching trays (30 x 30 x 10 cm) lined with paper-towelling and maintained at 37-38°C and 95% relative humidity until hatching on day 21 of incubation. Apart from one group (that was kept in a darkened incubator, 0 lux; see Chapter 7, page 172), the embryos were exposed to light (approximately 100-200 lux) from day 17 of incubation until hatching. All of the embryos were exposed to odours that are normally present in the incubator (e.g. odours of the other chicks, paper-towelling or from the plastic incubator trays). Some of the embryos were exposed to additional odours, as part of the experimental procedures, during the latter part of incubation (as outlined in Chapter 9).

On day 21 of incubation the embryos were checked every hour and any chicks that had hatched were removed from the incubator and weighed. A coloured and numbered ring band (Kra-mar pet supplies, Villawood, NSW) was placed on the leg. The chicks were then placed into a small force draft hatching incubator for a further 18 h. Any chick assessed as being physically abnormal was removed at this stage. Abnormalities were found in no more than 1% of chicks.

HOUSING

Each chick was housed individually in a clean grey metal cage (21 x 21 x 31 cm) with a clear perspex front 18 h after hatching. This cage is referred to as the home-cage. Water was provided from a clear plastic water drinker with a yellow lip (Kra-mar pet supplies, Villawood, NSW) which was located towards the rear of the home-cage. The home-cages were placed on a layer of paper towelling and situated on a three-tier rack. The chicks were selected at random from either the upper, middle or lower tiers of the rack, to minimise potential differences between fear responses. This procedure was followed as it has been shown that laying hens housed on the top tier, compared to those housed on lower tiers, show a decrease in approach and activity towards a novel object when placed in an unfamiliar situation (Jones, 1984; 1985; 1987b). Warmth and illumination were provided by 40 W bulbs and the temperature within the home-cage was maintained at 29-33°C. Feeding and drinking were encouraged by sprinkling 5 g of chick starter mash (Fielders, Tamworth, NSW) in front of the chick and by dipping its beak into water. Thereafter the chicks remained undisturbed for 2-6 h before testing with free access to food and water. An overview of the procedures used in these experiments is outlined in Figure 2.1.

No restriction was placed on the time when chicks were tested (even though this often meant that the chicks were tested at night) but instead the chicks were tested between 20-26 h post-hatching. The age of the chicks, rather than the time they were tested, seemed particularly important as, after hatching, the chick develops rapidly and precisely timed changes in behaviour are known to occur during the first week

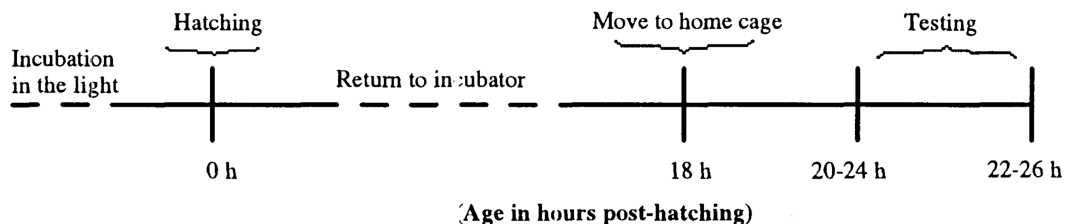

Figure 2.1 An outline of the timing of events in the behavioural experiments. Each chick was housed at 18 h post-hatching and tested individually between 20-26 h post-hatching.

post-hatching (Workman and Andrew, 1989; Dharmaretnam and Andrew, 1994). Furthermore, cyclical patterns in locomotor activity during the first 96 h post-hatching appear to depend on the chick's age rather than the time of day (Miller, 1980), although this is not the case for learning by older chicks. Chicks tested on the pebble floor task, in which the chick is said to learn if it can discriminate between pebbles and grains of food (Rogers, 1974), at 9 days post-hatching peck principally at grains during the day but at night they peck equally at pebbles and grains (Reymond and Rogers, 1981).

To determine if there was an effect of time of day on the chicks' responses in the present experiments, the pecking and head shaking responses during the first training trial (see later for the details of the test) were compared at different times of day. These data (presented in Figure 2.2) indicated that there was no significant time of day effect on the amount of pecking but there appeared to be some cyclical activity in the chicks' pecking responses with several peaks occurring at 4-6 h intervals. It is curious as to why such a regular cyclical pattern of pecking would exist in day-old chicks. This may reflect an interaction of additional factors such as hatching position within a batch or sleep-wake cycles. Furthermore, it can be noted that the level of head shaking was relatively low overall. Therefore, each of the experiments contained groups of chicks that had been tested at different times of day.

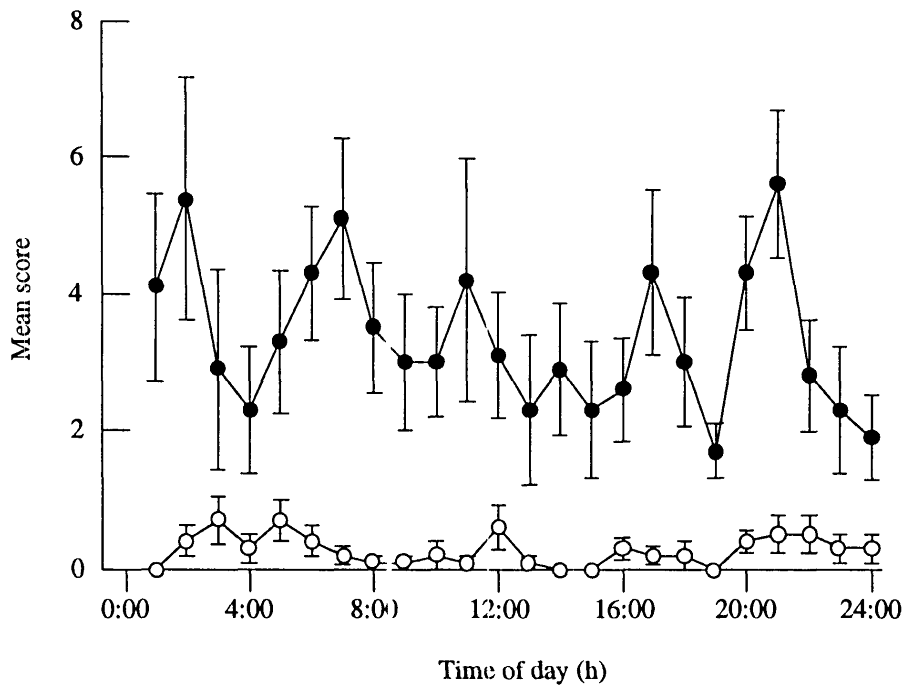

Figure 2.2 Effects of time of testing on the responses of separate groups of chicks each tested once during a 24 h period. The mean ± SEM pecking (●) and head shaking (○) responses is indicated for the first 10 chicks tested during each hourly interval (N=240). The scores were obtained during the first training trial and were selected *post hoc* from the entire data set obtained from this thesis. There was no overall significant time of day effect on either the pecking (Kruskal-Wallis test: *KW*=23.40, *df*=23, *P*=0.44) or the head shaking responses (*KW*=29.58, *df*=23, *P*=0.16). To further minimise other potential differences in responding, each experiment included chicks that had been tested at different times of day.

TESTS OF OLFACTION

Two different tests of olfaction were designed. One test required pecking at beads, as young chicks will readily peck at small visually conspicuous objects (Hogan, 1971). The stimulus was designed such that, when the chick pecked at the visual stimulus, it was exposed to an odour. Therefore, each chick had to respond to olfactory and visual cues. The other olfactory test did not involve the presentation of a visual stimulus. Instead, odorants were delivered to the chick housed in a sealed, glass chamber situated within a grey-metal cage of the same dimensions as the home-cage. The behaviour of the chick was observed following the presentation of odorant in a preliminary trial. However, this method was not particularly successful for obtaining responses to odorants by chicks as they demonstrated high levels of peeping and spent considerable amounts of time scrabbling at the sides of the cage in an attempt to escape from the glass chamber. The "glass chamber" test was not pursued, in part, because of some difficulties associated with it but primarily because early results using the bead test suggested that it was a more appropriate test with which to examine the chicks' responses to olfactory cues. All of the experiments reported in this thesis used the first of these tests (bead test) and it is described in detail below.

Visual components of the testing stimulus

The visual components of the testing stimulus are illustrated in Figures 2.3 and 2.4. They included a coloured plastic bead (4 mm diameter, Crown Fox, Japan) affixed to a white opaque plastic sample cup (500 µl COBAS sample cups, Roche, NSW) which was attached to the end of a 250 mm long glass tube (4 mm outside diameter; 3 mm inside diameter). The range of bead colours used was white, red, dark blue, light blue, dark green, light green, yellow and chrome (silver-grey metallic finish). A white bead was used during the training trials only (see below). The specific bead colour(s) used in each experiment are outlined in the relevant chapters.

It was necessary to make several modifications to the sample cups before they could be used for testing (see Figure 2.3). The base of the sample cup was tapered so that the bead could be inserted easily. The walls were perforated with fifteen evenly

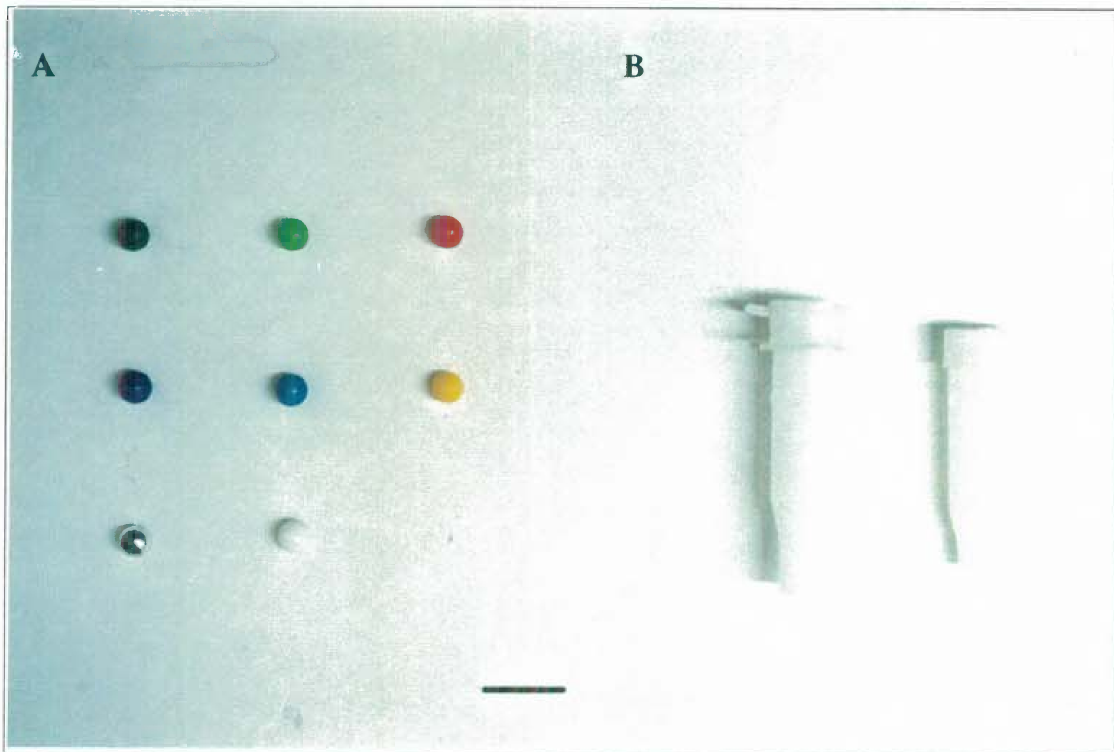

Figure 2.3 Examples of the coloured beads (A) and modified sample cup (B) used in the behavioural experiments. Note that several modifications were made to the sample cup (depicted on the left) before it could be used for testing (depicted on the right). Scale bar = 10 mm.

Figure 2.4 Presentation of the assembled apparatus used in the bead task. The visual components of the testing stimulus were assembled by affixing a bead to the sample cup and then inserting a 250 mm length of glass tubing before presentation to the chick.

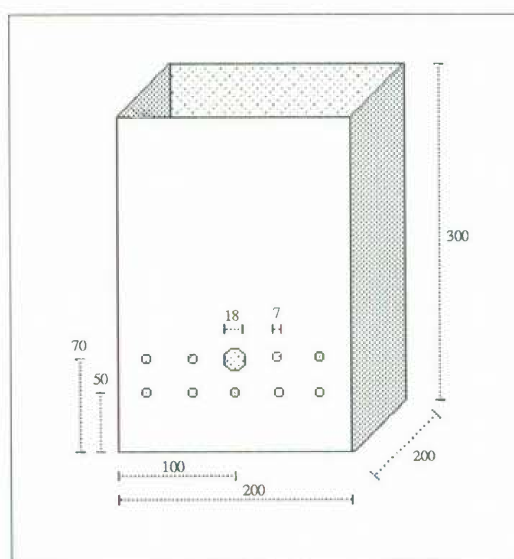

Figure 2.5 This is a representation of the testing cage used for the behavioural experiments. Dimensions are in mm.

spaced 0.5 mm diameter holes to allow for adequate dispersal of odour, and the lid from the sample cup was removed so that it could be positioned on the end of the glass tube before testing (see Figure 2.4).

Chicks were tested in a grey metal cage (see Figure 2.5), referred to as the testing cage, that had the same dimensions as the home-cage. The front of the cage consisted of a metal sheet with 9 holes (7 mm diameter) spaced evenly in two rows approximately 50 and 70 mm from the floor of the cage. The testing room was visible through these holes and they served to direct the chick's attention towards the front of the cage.

The testing stimulus was introduced into the testing cage through a tenth, centrally placed hole (18 mm diameter). The testing cage was illuminated with a single 40 W bulb. A video camera (NV-MS1, Panasonic) was located directly above the testing cage, attached to an external monitor and all of the tests were recorded on video tape. To prevent the chick from seeing the experimenter, the image from the monitor was used to guide the bead to a central position 5 mm from the tip of the chick's beak.

Preparation of odorants

A range of single, reagent-grade odorants was used in the experiments reported in this thesis and these are outlined in Table 2.1. The purity of these odorants, referred to in the table, provides an indication of chemical purity, or the level of contamination with

Table 2.1 Characteristics of the single, reagent-grade odorants as supplied by Aldrich (USA)

Substance	Purity	Density	Boiling point (°C)	Vapour pressure (mm Hg at 25 °C)	Molecular weight	Formula	Structure
Eugenol	99%	1.07	254	0.03	164.2	$C_{10}H_{12}O_2$	
Geraniol	98%	0.89	229	0.05	154.24	$C_{10}H_{18}O$	
Cineole	99%	0.92	176	1.96	154.24	$C_{10}H_{18}O$	
Methyl anthranilate	99%	1.17	256	0.04	151.16	$C_8H_9NO_2$	
(*R*)-(+)-Limonene	97%	0.84	176	2.03	136.23	$C_{10}H_{16}$	
iso-Amyl acetate	98%	0.88	142	5.32	130.19	$C_7H_{14}O_2$	
Allyl sulfide	97%	0.88	138	8.89	114.21	$C_6H_{10}S$	$CH_2=CH-CH_2-S-CH_2-CH=CH_2$
Ethyl alcohol	70% in H_2O	0.79	78	56.11	46.10	C_3H_6O	
Ammonia	29% in H_2O	0.80	-	-	17.03	NH_3	

chemical impurities. The chemical characteristics and molecular structure of each of the odorants (Weast, 1986) is also presented in the table. Although the purity of these chemicals is known this does not mean that they are pure odours, in the field a 'pure odour' is referred to as one that stimulates the nasal epithelium only, in a way that is uncontaminated by trigeminal activity (Doty, 1995; see also Chapter 1). In this thesis, therefore, the term 'odour' will be used, unless specified otherwise, to describe the volatile compounds which result in stimulation of chemoreceptors, possibly including both olfactory receptors and free endings of the trigeminal nerve, within the nasal cavity. While recognising that trigeminal stimulation may be involved, the responses will be referred to as 'olfactory'. Also, the terms odour and odorant will be used synonymously. The chemicals used as odorants will be referred to as 'single odorants'.

Besides the single odorants, a range of complex (or mixed) odorants were used (reported in Chapters 5 and 9). Each of the mixed odorants was from a substrate of organic origin, such as wood shavings or chick feed. These odorants are likely to consist of mixtures of tens, if not hundreds, of single odorants. Some of the probable main single odorants contributing to the odour mixtures are detailed in Table 2.2.

Table 2.2 Characteristics of the mixed odorants used

Substance	Source	Probable main odour components
Starter mash	Grain-based diet: Fielders	Grains and grasses
Feathers	Isa brown layers: Poultry shed	Wax diesters, sterols, steryl esters, fatty acids [a]
Wood litter	Shavings from mixed hard and soft woods: University of New England	Pinene, terpenes, tannins, resins
Faeces	Isa brown layers: Poultry shed	Fatty acids, phenols, indoles, methylamines, hydrogen sulfide, methane, *ammonia* [b]
Blood	1-day-old white leghorn x australorp chicks	hormones, uric acid, plasma proteins

Source: *a* (Wertz *et al.*, 1986), *b* (Nielson *et al.*, 1988; ammonia is indicated in italics as although it is a volatile component of most animal faeces it has been found that, at least in humans, at these concentrations it contributes little to the overall perceived odour intensity).

Two different types of odorant delivery system were used. In some experiments the odorant was applied to a piece of cotton wool within the test stimulus, referred to as static olfactometry, while in other experiments the odours were delivered by an olfactometer, referred to as dynamic olfactometry. The methods for these two procedures will be described separately.

Static olfactometry

The test stimuli were prepared by applying a known volume of a single odorant to a clean piece of cotton wool inside the sample cup. Some experiments used log dilutions of odorant made with 70% ethyl alcohol (these methods are detailed in Chapter 3). Other experiments used the addition of either 1, 10 or 100 µl of undiluted odorant to the sample cup. The odorant could diffuse through the holes in the wall of the sample cup to be present in the air surrounding the bead. The amount of odour delivered to the chick is referred to as the volume of odorant applied to the sample cup. Although the concentration of the odour that diffused into the air surrounding the bead was not measured directly, it is affected by properties of the odour, the temperature and the external pressure (Dravnieks, 1975). Furthermore, the rate of odour diffusion (J) is directly proportional to the change in concentration per unit distance, $(C_1 - C_2)/\Delta x$ or the concentration gradient, the diffusion coefficient of the odorant in air (D) and to the cross sectional area of odorant exposed to air (A) according to Fick's diffusion law (Giancoli, 1985);

$$J = DA \frac{C_1 - C_2}{\Delta x}$$

The concentration of odour that the chick is exposed to depends not only on the rate of diffusion of the odour in air, as mentioned above, but also on the chick's nasal air flow. An increase in the distance between the chick and the odorant source means that the chick would be exposed to a lower concentration of odour due to the odour concentration gradient. Of particular importance, therefore, is the assumption that the chicks breathed during the bead presentation. Several observations were made to

determine the chick's breathing patterns. This was performed by observing the cycles of abdominal movement as an indication of respiratory frequency.

The respiratory frequency of six chicks was observed in the home-cage before testing. The same chicks were then placed in the testing cage and presented with a white bead attached to an unscented sample cup. The number of breaths before and after the presentation of the stimulus was recorded (see Table 2.3). While they were sleeping (lying with their eyes closed in the home-cage) they made approximately 40-55 breaths min^{-1}. After they had been placed into the testing cage and presented with an unscented bead their respiratory frequency increased to 53-84 breaths min^{-1}. Of those chicks that pecked the bead (n=5) 2-6 breaths were made during a period of 2-5 s before pecking the bead. Therefore, the chick inhales while it is close to the bead and would thus inhale odorant vapour, but there is no way of knowing the odorant concentration that reaches the nasal cavity.

To avoid the presence of lingering odours in the testing room the stimuli were prepared in a room separated from the testing room. Disposable latex gloves were used when handling the odours. Each stimulus was placed into a sealed scintillation vial and stored at 4°C for no longer than 24 h. The stimuli used at test were randomised with respect to the storage time of the odour. Before presentation, the stimuli were warmed to room temperature (26-29°C). The stimuli were always kept in the sealed vials, apart from when they were presented to the chick.

Dynamic olfactometry

The second method of odour delivery used an olfactometer that mixed the odorant with a stream of air. The system was constructed of glass and silclear tubing (Bacto Laboratories, Australia). Its design is illustrated in Figure 2.6. Commercial grade air was purified by passing it through a column of activated charcoal and then a column of silica gel (BDH Chemicals, Kilsyth Victoria). The air flow was controlled by needle valves and monitored by two inline flow meters. The air flow rate at the delivery tube was maintained at 250 ml min^{-1}.

Table 2.3 Respiratory frequency of six chicks observed in the home-cage and in the testing cage

Chick number	Sleeping in home-cage prior to test	Testing cage		
		Before bead presentation	During bead presentation	Before pecking bead ‡
34	40	67	84	120
38	51	53	78	90
32	45	53	60	60
26	45	75	54	48
50	46	47	60	*did not peck*
20	55	66	53	60
Mean ± SEM	47.0 ± 2.1	5[illegible].5 ± 7.3	64.8 ± 5.3	75.6 ± 13.1

The values tabulated above indicate the respiratory frequency in breaths min^{-1}. These data were obtained by direct observation of the same group of chicks while 'sleeping' in the home-cage as well as before and after the presentation of a white bead in the testing cage. ‡ The number of breaths taken in the time between the introduction of the bead into the testing cage and the chicks' first peck at the bead are also indicated. Note that chicks had a higher respiratory frequency while they were in the testing cage, compared to sleeping in the home-cage.

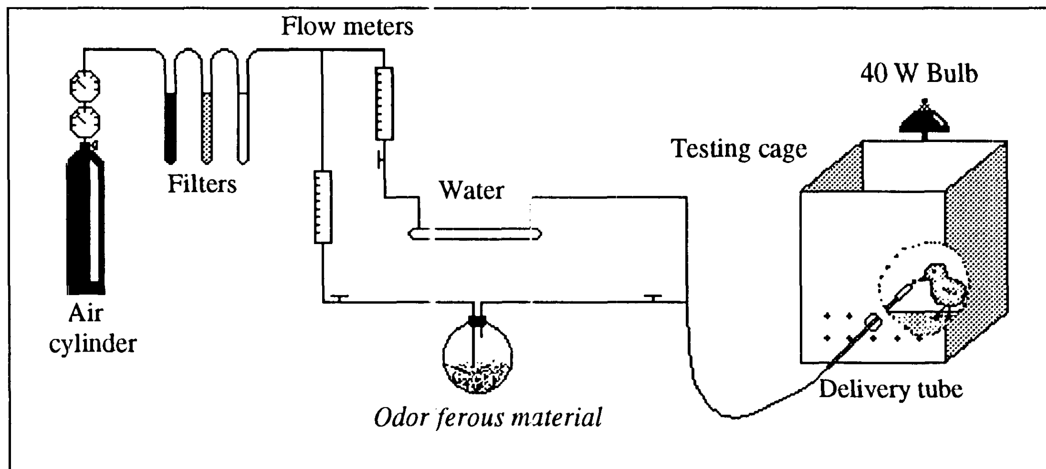

Figure 2.6 Schematic diagram of the olfactometer used to generate air saturated with odorant vapour. The system was constructed of glass and silclear tubing. Air flow was varied with inline flow meters and needle valves. Clean air was produced by passing air over distilled water. This could be mixed with a separate line that contained air saturated with the vapour of an odorant. The system could be connected to the testing stimulus and presented to the chick, as shown.

Saturated vapour was generated by passing filtered air at a constant flow rate over the odorant. The odoriferous material was contained within a 500 ml round glass flask, which was sealed with a stopper, and connected to the system through an inlet and an outlet tube. Humidified, clean air was generated by passing air over 26 ml of distilled water contained in a folded 2.6 cm diameter, 26 cm long glass tube. The containers were maintained at room temperature (26-29°C).

The concentration of odour delivered to the chick, using dynamic olfactometry, remains constant. That is, the chick would inhale a relatively constant concentration of odour (in parts per million, ppm) at a distance (x) from the bead. With static olfactometry, which relies on diffusion, the concentration of odour decreases with increasing distance from the bead (see Figure 2.7). However, as with static olfactometry, the concentration of odour reaching the olfactory epithelium depends on whether the chick is inhaling or exhaling when it is close to the bead and on the volume of air that is inspired.

Procedure

Training began when each chick was 20-24 h post-hatching and involved the presentation of a stimulus for two 20-s trials in the testing cage. The stimulus used in the training trials was a white bead attached to an unscented sample cup. The training trials were used to familiarise the chicks with the testing apparatus. These were followed by a series of testing trials in which stimuli were presented for 10 s. The details of specific testing procedures are outlined in the relevant chapters. The stimulus was presented to the chick 10 s after it had been placed in the testing cage. Each training and testing trial was separated by a 10 min interval during which the chick was returned to its home-cage. In all of the trials the number of pecks and head shakes were recorded during each of the 20 s (training) and 10 s (testing) periods.

Behaviours scored

Pecking

Pecking was scored when a chick pecked at the bead, but not when pecks were

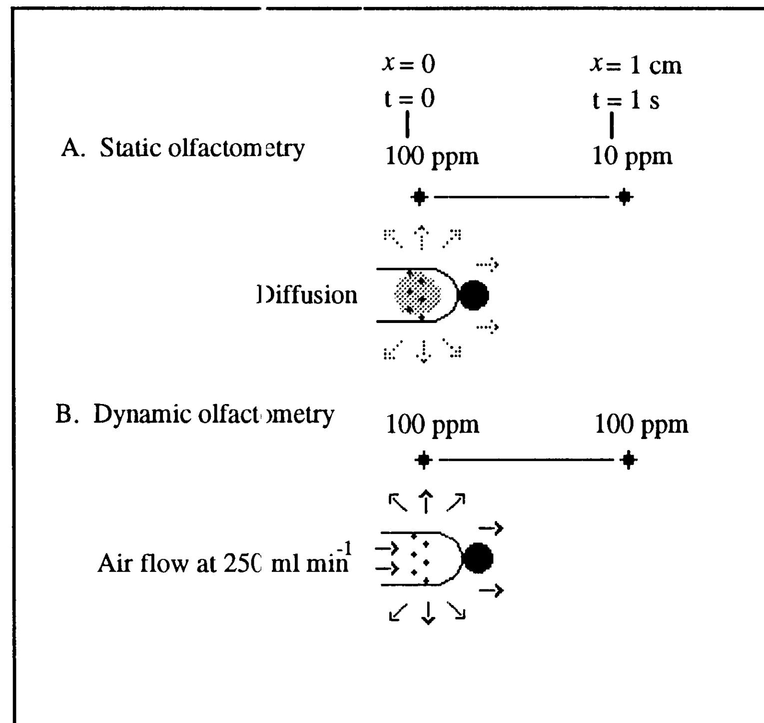

Figure 2.7 Diagrammatic representation of odorant delivery by static (A) and dynamic (B) olfactometry. For comparison between the two methods, the same concentration of odorant (say 100 ppm) is contained in 1 ml of air at the sample cup. At a distance (x) of, for example, 1 cm from the sample cup and at some time after the onset of odour delivery (say t=1 s) the concentration of odorant will be higher using dynamic olfactometry than by simple diffusion using static olfactometry.

directed at the sample cup, testing cage or floor (less than 5% of the chick's pecked the sample cup). The bead was held 5 mm from the tip of the chick's beak. Chicks demonstrated aimed, ballistic pecking at the bead with either an open or closed beak; sometimes they would mandibulate the bead for a brief time (< 1 s). A new peck was scored, after initial contact with the bead, only when the chick made a separate ballistic movement of the beak towards the bead.

Head shaking

A bout of head shaking is a number of rapid, lateral movements of the head and it typically has a duration of 0.1 - 0.4 s. A single bout of head shaking was defined as at least 3 alternating, lateral movements of the head within 0.1 s. A new bout of head shaking was scored when the head remained stationary for at least 0.4 s. Head shaking was scored only while the bead was within the testing cage.

OCCLUDING NOSTRILS

A commercially available, 'unscented', depilatory wax containing gum resin, beeswax and camauba wax was used to occlude the nostrils (Tender Beauty Products, Newcastle, NSW). No additional odour (scent) was added to the wax preparation and it was thus referred to as 'unscented' That is not to say that the wax was completely odourless as the resin and wax had a distinct odour. The term 'unscented' is not used synonymously with 'odourless' but rather means that no additional odour has been added. The wax preparation was relatively easy to apply and, when applied to the chick's beak, remained in place for at least 24 h (see Figure 2.8).

The wax was softened by placing it into a glass vial in a water bath maintained at 70°C and then it was applied to one or other of the chick's nostrils using a small metal spatula such that it completely covered the external opening and a small portion of the surrounding beak. The application of a thin layer of the wax (approximately 2 mm, see Figure 2.8) to the nostrils did not obscure the chick's vision. In most of the experiments that required nostril occlusion the chicks were returned to their home-cage for 10 min before testing. However, chicks in one group, reported in Chapter 8, had both nostrils

Figure 2.8 Photograph of chick with one nostril occluded by a wax preparation. Note that the wax did not obstruct the chick's vision.

occluded approximately 2 min before testing. Some chicks vocalised and struggled when the wax was applied but as soon as they were returned to their home-cage they did not appear to be unduly stressed. Typically, their behaviour included a brief period of head shaking and preening in an attempt to remove the wax plug followed by pecking at grains of food, sitting or sleeping.

STATISTICAL ANALYSIS

A Kolmogorov-Smirnov goodness-of-fit test showed that the scores for pecking and head shaking were not normally distributed. Moreover, plots of the mean pecking and head shaking scores against their standard deviation demonstrated that the variances were not homogeneous. No transformations, including a standard log transformation for right-skewed data (Martin and Bateson, 1994), were able to normalise the data. Therefore, non-parametric statistical procedures, unless specified otherwise, were used throughout this thesis. Although the scores from the training trials were analysed separately from the testing trials, the same statistical procedures for each comparison were made using the SPSS^{X}/windows statistical package.

A Kruskal-Wallis one-way analysis of variance by ranks (*KW*) was applied to data arising from independent groups of chicks. Data showing significant heterogeneity was further analysed using two-tailed Wilcoxon-Mann-Whitney tests (z; previously referred to as the Mann-Whitney U-test; Siegel, 1956). The Friedman two-way analysis of variance by ranks (F_r) was used to determine the effects of repeated testing on the pecking and head shaking scores. Significant differences were analysed *post hoc* with the Wilcoxon signed ranks test (z), two-tailed. In addition, relationships between variables were examined using Spearman rank order coefficient of correlation (r_s). All non-parametric statistical tests were used according to the procedures described by Siegel and Castellan (1988) and two-tailed probabilities were used for all tests with two samples. Where required, additional statistical procedures were used and these are described in the relevant chapters. Statistical values with a probability value (P) of ≤ 0.05 were considered to indicate significant differences between groups.