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Tinbergen's fourth question, ontogeny: sexual and individual differentiation

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Abstract

Based on Tinbergen's view of the study of behavioural development we describe some recent advances and their importance in this field. We argue that the study of behavioural development should combine both proximate and ultimate approaches, and can help to understand how early subtle environmental factors shape consistent individual variation both between and within sexes. This is illustrated by reviewing the profound effects of incubation temperature on the development of brain and social behaviour in the leopard gecko, a species with temperature-dependent sex determination, and the effects of early exposure to steroid hormones on social behaviour in rodents and especially birds. Both are maternal effects: incubation temperature can be partly determined by the nest site where the mother deposited her eggs, while in both oviparous and viviparous vertebrates maternal hormones reach and influence the embryo. In the gecko, incubation temperature affects sexual and aggressive behaviour, growth, the hypothalamus-pituitary-gonadal axis, as well as the size, connectivity and metabolic capacity of certain brain areas. In this way not only is the gonad type determined, but so too is the morphological, physiological, neural, and behavioural phenotype established that explains much of within-sex variation. In rodents, maternal hormones affect similar aspects. In avian species, maternal hormones, deposited in the eggs, vary systematically between and within clutches and have both short- and long-lasting effects on competitive behaviour. Evidence suggests that mothers adaptively adjust hormone allocation to the environmental context. In addition, we discuss some effects of postnatal experience on behavioural development in geckos, mice and bird species. Our results also illustrate how the study of animal models other than rodents can help in understanding important developmental processes.

Keywords

behavioural development; bird; cytochrome oxidase; individual differentiation; lizard; maternal androgens; temperature-dependent sex determination

INTRODUCTION

Ontogeny of behaviour, the development of behaviour within the individual, was the fourth pillar of Tinbergen's (1963) treatise that marks the new synthesis of modern ethology. It was his contribution to Huxley's (1914, 1923) 'three major problems of biology'; namely causation, survival value, and evolution. Here he recognised that while development had long been a subject of study by embryologists, for behaviourists it had become "a field in which there is a real clash of opinion." The controversy reflected the different approaches practised by students of behaviour in western Europe and North America.

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The European approach was strongly influenced by Lorenz, one of the founding fathers of ethology. Lorenz is perhaps best known for his discovery of sexual imprinting; namely that the young of precocial birds would, if presented during a certain window of time post hatching, follow any conspicuous stimuli as if it were their mother, despite the fact that the stimulus did not have the slightest similarity with a conspecific, and that this attachment even transformed in adulthood to sexual preferences for that stimulus. This, and the development of species-specific motor patterns (Lorenz and Tinbergen, 1938), led him to the proposition that the development of certain behaviours is pre-programmed by genes and independent of experience. This view was rejected vigorously by behavioural scientists in North America, most notably Hebb (1953), Lehrman (1953), and Beach and Jaynes (1954). Lehrman's 'Critique of Konrad Lorenz's Theory of Instinctive Behaviour' was particularly influential, in part because of the journal in which it appeared, the recognised skills of the author in the observation of animal behaviour, and the personalised nature of the writing. In this view the subtle interplay of internal and external processes were considered essential in the development of any behaviour, including imprinting. Such diametrically opposed opinions led to Tinbergen's understatement that "there is no agreement about the nature of the problems involved, and while the methods applied by psychologists and ethologists begin to resemble each other so closely as (in some instances) to be indistinguishable, the interpretation of the results gives rise to much discussion" (Tinbergen, 1963, p. 423).

Tinbergen recognised this dichotomy as "heuristically harmful." Indeed, the nature of scientific discovery, based as it is on cumulative knowledge, means that preconceptions often poison perception, in this case leading to intransigence. Faced with the delicate situation of writing a paper in honour of his friend's 60th birthday and, at the same time, disagreeing with Lorenz's perspective of behavioural development, Tinbergen sought to elevate the debate and proposed a new paradigm for further research. "If I were to elaborate this further I should have to cross swords with my friend Konrad Lorenz himself ... but this is not the occasion to indulge in swordplay, and I prefer to continue with my sketch of the procedure which seems to me more fruitful" (Tinbergen, 1963, p. 425).

"I believe that this discussion has been and is still being bedevilled by semantics, and that it would be helpful if, instead of discussing the justification of the use of words such as "innate" and "acquired", of "instinct" (and "instinctive") and "learning", we could return to a statement of the phenomena to be understood and the questions to be asked — indeed I think this is imperative" (Tinbergen, 1963, pp. 423–424).

Tinbergen (1963) proposed that the suitable subject of study of behavioural ontogeny should be the "change in behaviour machinery during development." This meant that "the phenomenon (change in behaviour machinery) has to be described; the problem is, how are these changes controlled? As a first step one distinguishes between influences outside the animal and internal influences." Unfortunately, like others before and after him, Tinbergen's solution did not quell the nature/nurture debate, perhaps because of the human tendency to succumb to the seduction of simplicity and dichotomies in lieu of the reality of complexity.

In real life, genes, other internal factors, and external factors continuously interact with each other. And although differences in behaviour between individuals can be attributed to differences in the contribution of one of these factors, each factor is important and indispensable for the development of individual behaviour. This proposition has now been firmly established in the forefront of ontogenetic research, despite ongoing discussions and misconceptions that still appear both in scientific and especially popular papers. We will discuss some examples of behavioural development within this integrative view. In keeping with the suggestion of Tinbergen, we will not indulge in semantics, but try to illustrate how genetic, ecological,

physiological, and social processes, and the integration among these, shape the development of social behaviour.

The way Tinbergen formulated the study of behavioural development suggests it to be the study of causal mechanisms. However, young organisms have to be adapted to the environment in which they are born, and because they are different from adult conspecifics they need their own adaptations. Hence, behavioural development consists of so-called ontogenetic adaptations, and is a product of evolutionary history. In fact, all of the other of Tinbergen's 'Why' questions detailed in his 1963 paper, namely description, causation, function and evolution, can and should be applied to the study of behavioural development. It is our view that in behavioural biology questions about the causation of development (changes in the underlying machinery of behaviour as Tinbergen called it) should be inspired by considerations about function: what are the problems that have to be solved by developing organisms to survive and reach maturity? We will address functional aspects of development throughout this paper.

At the time Tinbergen wrote his paper, ethologists were mainly concerned with the development of species-typical behaviours. Differences in behaviour between individuals of the same species were mostly interpreted as noise around an adaptive mean. Nowadays it has become clear that behavioural differences between individuals of the same species and sex may reveal important biological information. Therefore, we will describe some mechanisms by which differences in the social behaviour between males and females as well as that of individuals of the same sex, can develop.

Mothers can influence the development of their offspring not only by genomic, but also by non-genomic effects. The advantage of the latter is that they can be adjusted to the situation in which the female is reproducing and in which the young will be reared. Interestingly, such non-genomic effects may be carried across generations that may generate patterns that may seem to be, but in fact are not, heritable. Two clear examples of this are the effects of incubation temperature and maternally-derived hormones, and we will focus on these. We will discuss the influence of these factors, as well as (their interaction with) social experience, on the development of behaviour during three different age classes: prenatal, early postnatal or juvenile stage, and adulthood.

We are, within the tradition of early ethologists, convinced that the use of different animal models, other than the classical laboratory rodent, can be of great help to the understanding of behavioural ontogeny. Therefore we will discuss examples of a variety of animal taxa, such as reptiles, birds, and rodents.

THE EMBRYONIC ENVIRONMENT

Although the effect of developmental processes on behaviour often only becomes apparent after birth or hatching, the prenatal stage is very important for the developmental process itself. During this early stage physiological factors affect development of brain and behaviour, often during a sensitive phase and with irreversible effects. Such early processes can affect a whole array of behaviours. Clear examples of this are the effects of steroid hormones on sexual differentiation, affecting sexual and agonistic behaviour, and the effect of light on brain lateralisation in avian species (Rogers 2002), affecting both social and non-social behaviour. Therefore, most of our paper deals with this early phase in development. We will discuss two examples in some detail, the effect of incubation temperature on sex determination and sexual differentiation in the leopard gecko, and the effect of prenatal exposure to maternal androgens in birds. Both examples illustrate, each in its own way, the importance of the mother in shaping individual differences in behaviour in the offspring.

Sex determination and sexual differentiation

Temperature-dependent sex determination: the leopard gecko model—Mammals and birds have sex chromosomes. In other words, genetic sex and gonadal sex are inextricably linked. This genetic difference facilitates the study of sex differences and, indeed, one of the great success stories in genetics of the last century was the discovery of a gene on the short arm of the Y chromosome dictating whether the primordial gonadal ridge would develop into an ovary or a testis. In fact the process of sexual differentiation is very illustrative of how genes are translated to behaviour via the production of gonads, that determine in turn the hormonal milieu, which in turn determines adult sexual behaviour, a process with a lot of plasticity despite the strong influence of genetic factors.

However, the very nature of genotypic sex determination makes it difficult to distinguish epigenetic from genetic contributions to sexuality. Consider, for example, aggressive and sexual behaviours displayed by both sexes, but at different frequencies. To what extent are differences observed between adult males and females due to their differences in sex chromosomes, differences in the nature and pattern of hormone secretion, differences in non-genomic yet heritable factors such as maternal influences, or even sex-typical experiences? So, in animals with sex chromosomes, the genetic element typically cannot be separated from the epigenetic element. This is particularly important as the sex chromosomes may be involved in the sexual differentiation of the brain as well as the gonads (Arnold, 2002).

Ideally, studies of the development of sexuality would utilise animal models that exhibit sex-typical differences in the traits of interest, yet not have the complications arising from sex-specific chromosomes. In other words, a species that can illustrate how different environments can elicit different phenotypes from a particular genotype without the confound of sex-limited genes. Do such organisms exist in nature?

Fortunately, not all vertebrates have sex chromosomes. Indeed, there are many vertebrate species in which sex is determined not by sex chromosomes, but by the environment (Crews, 1993, 2002). One form of environmental sex determination is temperature-dependent sex determination (TSD). In TSD, gonadal sex is initially plastic but becomes fixed by the temperature of the incubating egg during the midtrimester of development (Crews, 1996). The Crews laboratory has been studying TSD both in terms of its causal mechanisms and its functional outcomes.

We have taken advantage of the TSD system to investigate how events early in life can act directly on body and brain to shape adult sexuality independently of the gonad and its hormones. In this work we have used a TSD animal, the leopard gecko (*Eublepharis macularius*). This species also lacks parental care, facilitating the interpretation of results of incubation temperature per se. In the leopard gecko, high and low incubation temperatures produce only, or mostly, females, while intermediate incubation temperatures produce different sex ratios (fig. 1). That is, 26°C and 34°C are female-producing incubation temperatures, whereas 30°C produces a female-biased sex ratio, and 32.5°C produces a male-biased sex ratio. It is important to note that incubation temperature and gonadal sex are not completely linked in TSD; in other words, this association is not as fixed as gonadal sex and genetic sex are in species with genotypic sex determination. Rather, the effect of incubation temperature and gonadal sex can be dissociated. Thus, the effect of gonadal sex can be determined by comparing males and females from the same incubation temperature and the effects of embryonic temperature can be determined by comparing males or females from the different temperatures. In other words, when sex differences are the focus, we compare males and females from within a particular incubation temperature, and when the development of sexuality is the focus, we compare males (or females) from the different incubation temperatures.

Temperature-dependent sex determination and the development of individual differences—The results of a variety of experiments indicate that incubation temperature does more than just establish the gonadal sex of the individual. That is, the temperature experienced during embryogenesis accounts for much of the within-sex variation observed in the morphology, growth, endocrine physiology, and aggressive and sexual behaviour of the adult (Crews et al., 1998; Crews, 1999; Rhen and Crews, 2002; Sakata and Crews, 2004). For example, males in general grow more rapidly and are larger than females. That is, males from the intermediate incubation temperatures are larger than are females from the same temperatures. However, females from the male-biased incubation temperature grow as rapidly and become as large as males from female-biased incubation temperature. The temperature experienced during embryonic development also determines the relative concentrations of sex hormones that the individuals exhibit as adults. In general there is a log unit difference in the androgen-to-oestrogen ratio between males and females. However, the endocrine physiology of the adult varies dramatically with the temperature experienced during incubation. In both sexes the androgen-to-oestrogen is highest in animals from the male-biased incubation temperature.

Incubation temperature also has a major influence on the nature and frequency of the behaviour displayed by the adult leopard gecko. In general, females usually respond aggressively only if attacked, whereas males will posture and then attack other males but rarely females. Females from a male-biased incubation temperature are significantly more aggressive toward males than are females from a low or female-biased incubation temperature. These same females show the maletypical pattern of offensive aggression. Similar effects of incubation temperature on aggressive behaviour have been documented in males.

Courtship is a male-typical behaviour. In a sexual encounter, the male will slowly approach the female, touching the substrate with his tongue or licking the air. Attractiveness is a female-typical trait and is measured by the intensity of a sexually active male's courtship behaviour. Females from a male-biased incubation temperature are less attractive to males than are females from lower incubation temperatures. Interestingly, attractivity in females from the high (34°C) incubation temperature is greater than that of females from male-biased incubation temperature and not different from that of low-temperature (26°C) females. The mating preferences of males are also influenced by incubation temperature as indicated in Y-maze tests. Males from the male-biased incubation temperature prefer females from a low incubation temperature (Putz and Crews, in press) (fig. 2).

Aggressive and sexual behaviours in the leopard gecko are modulated by sex hormones and incubation temperature also influences the ability of exogenous sex hormones to maintain or restore sexual and aggressive behaviours in both sexes. For example, we find that, depending upon their incubation temperature, adult males respond differently to hormone replacement therapy following castration and, as one would predict, incubation temperature modulates patterns of brain metabolism. Similar results have been found in females. This suggests that incubation temperature influences how the individual responds to steroid hormones in adulthood.

Because the nature and pattern of growth, hormone secretion, and behaviour are ultimately expressions of brain activity, it stands to reason that neural phenotypes must also exist and these might be sensitive to incubation temperature. How might incubation temperature during embryogenesis affect the brain of adult animals?

A unitary neuroanatomical framework

Given the wealth of evidence that in animals with genotypic sex determination certain limbic nuclei like the preoptic area and the ventromedial hypothalamus are sexually dimorphic, we

expected to find similar sex differences in the leopard gecko. It was a surprise to discover that there are no statistically significant sexual dimorphisms in either of these brain areas between males and females at those incubation temperatures that produce both sexes. There are, however, consistent differences across incubation temperatures. For example, the volume of the preoptic area is larger in both males and females from the male-biased incubation temperature compared to animals from the female-biased incubation temperature. The opposite pattern is found for the ventromedial hypothalamus. That is, the volume of the ventromedial hypothalamus is larger in females from low incubation temperatures compared to females from the male-biased incubation temperature. Other research has shown that if males from different incubation temperatures are castrated and given equivalent amounts of sex steroid hormones, the difference in agonistic behaviour between the different types of males persist, suggesting that incubation temperature of the embryo may directly organise the development of brain nuclei independent of gonadal sex in a manner similar to that for body growth.

A great deal has been learnt from studies in which brain areas have been destroyed or chemically or electrically stimulated, but there are limitations to what they can tell us about behaviour-brain relationships. While there is no disagreement that complex behaviours must reflect complex patterns of neural activity involving many areas of the brain, how to visualise such coordinated patterns of brain activity has been a challenge. One approach has been functional brain mapping using methods that include, but are not restricted to, cytochrome oxidase histochemistry, immediate early gene expression, and 2-deoxyglucose uptake that correlate patterns of behavioural expression with patterns of activity in various brain nuclei.

Cytochrome oxidase is a terminal enzyme of the electron transport chain located in the inner mitochondrial membrane and catalyses the transfer of electrons to oxygen to form water and ATP. Thus, cytochrome oxidase is a rate-limiting enzyme in oxidative phosphorylation, the major pathway in brain metabolism. Consequently, the abundance and activity of cytochrome oxidase activity in a brain area is a measure of the metabolic capacity of that brain region and reflects the metabolic history of an area. In other words, the activity of cytochrome oxidase determines the amount of ATP available in a neuron, thereby constraining the amount of activity a neuron can sustain and can serve as a marker of metabolic capacity in brain nuclei (Gonzalez-Lima, 1992). Cytochrome oxidase histochemistry is not like 2-deoxyglucose autoradiography or c-fos immunocytochemistry, which provide information on evoked or immediate activity; instead, cytochrome oxidase reveals long-term changes in brain activity.

Sexual behaviour is particularly amenable to the study of behaviour-brain relationships. A large number of experiments have identified the nuclei of the limbic forebrain to be critically involved in the display of sexual behaviour. Further, a variety of methods have revealed them to be interconnected, to contain sex steroid hormone receptors, and to be sexually dimorphic in their volume and synaptic organisation as a consequence of the nature and frequency of sex steroid hormones secreted perinatally. Importantly, these properties appear to be evolutionarily conserved. For example, in both mammals and reptiles metabolic activity in limbic areas reflects the capacity to display sociosexual behaviours and, in turn, that differences in metabolic activity in these areas reflect individual differences in the propensity to display social behaviours (reviewed in Crews, 1992; Sakata et al., 2001).

The possibility that these limbic nuclei might form an integrated neuronal network with overlapping functions that subserve all sex steroid hormone-modulated social and reproductive behaviours, has been considered previously, most recently by Newman (1999). Thus, the concept that social and reproductive behaviours may “emerge from the activity of a unitary neuroanatomical framework” in the brain (Newman, pp. 252–3), and not simply the product of activity of a single brain area(s), complements more traditional approaches of mapping different behavioural functions on subnuclei in these brain areas. Further, Newman suggested

a graphical representation of the data generated in studies of metabolic activity that would allow one to see how nuclei in this network may differ in different behavioural states. It is our opinion that her proposal represents a marked improvement on the traditional tabular form of data presentation that makes it difficult to detect the relationships among the various nuclei measured. While Newman proposed hypothetical representations, Crews has recently applied and expanded this method as it relates to the neural landscape of metabolic activity of the limbic nuclei associated with social behaviour. This conceptualisation is similar to both Waddington's developmental landscape during embryogenesis, and the peaks and valleys of a fitness landscape in theoretical biology.

We have been interested in how experience might affect the neural substrates of aggressive and sexual behaviour in leopard geckos. Extending Newman's concept, we have mapped which brain areas mediate experience-dependent changes in behaviour. For example, we find embryonic experience influences both between-sex and within-sex differences in the limbic landscapes. Relative to females, males on average have greater cytochrome oxidase activity in the preoptic area (Coomber et al., 1997). The complement is also true; that is, relative to males, females on average have greater cytochrome oxidase activity in the ventromedial hypothalamus. But incubation temperature is an important determinant in both sexes and these differences correlate well with behaviours exhibited by animals from different incubation temperatures. For example, males from the male-biased incubation temperature are more aggressive and have greater cytochrome oxidase activity in the anterior hypothalamus, septum, and the nucleus sphericus (homolog of the medial amygdala) compared to males from the female-biased incubation temperature (fig. 3). As might be expected, females from the female-biased incubation temperature have greater cytochrome oxidase activity in the ventromedial hypothalamus compared to females from the male-biased incubation temperature. As mentioned previously, there is a significant increase in aggression in females from higher incubation temperatures. Analysis of females from different incubation temperatures reveals that cytochrome oxidase activity increases in brain nuclei associated with aggressive behaviour as a function of incubation temperature in a manner that parallels the differences in aggression among females from different incubation temperatures.

Hormone mediated maternal effects and individual differentiation

Sibling effects in mammalian species—As we have seen above, hormones play a key role in the differentiation between the sexes. Differences in early exposure to sex steroid hormones produce permanent differences between males and females in morphology, brain and behaviour. Such permanent effects of early hormone exposure, inducing structural changes in the brain, are called organising effects. Part of these effects involves the sensitivity of the brain and other organs to the activating effects of hormones later in life. So far we have been discussing the effects of hormones produced by the embryo itself. However, the embryo is also exposed to steroid hormones from other sources, namely those of the mother, and in litter bearing species, of their siblings. This can have important consequences for development.

In litter-bearing mammals the embryos are positioned closely beside each other in the uterus. At some time during development, these embryos produce sex steroids that also reach the neighbouring foetus. As a consequence, the sex of an individual's neighbours in the uterus can influence its physiology and behaviour in adulthood (vom Saal et al., 1999). That is, the endocrine microenvironment during foetal development modifies the physiology, behaviour, and neurochemistry of the individual. Thus, a female foetus located between two males (a 2M female) is exposed to higher levels of androgen produced by the neighbouring males compared to a female foetus located between two females (a 2F female). Behavioural effects of this early endocrine milieu have been demonstrated in pigs, mice and gerbils. Largely through the work of Mertice Clark and Jeff Galef on the latter we have learned that as adults these 2M females

have lower oestrogen and higher testosterone levels, have a late onset and a long oestrus cycle, have a masculinised phenotype, are less attractive to males and more aggressive to females, and produce litters with significantly more male-biased sex ratios relative to 2F females (Clark and Galef, 1995). Effects of the intrauterine position in males have also been found. In addition, Howdeshell and colleagues (1999) Howdeshell and colleagues (2000) recently demonstrated that the foetus's intrauterine position also influences its sensitivity to exogenous hormones in adulthood. Such effects might also be relevant for explaining individual differentiation in twins in our own species.

In collaboration with Clark and Galef the Crews laboratory has demonstrated that the metabolic activity of brain nuclei can also vary according to uterine position. In gerbils, the sexually dimorphic area of the preoptic area is responsible for copulatory behaviour in males and, as females differ in their sexual behaviour according to intrauterine position, this area is likely to be involved in their behaviour as well. Cytochrome oxidase histochemistry reveals long-term changes in the metabolic capacity in the sexually dimorphic area of the preoptic area, with 2M females having greater activity compared to 2F females (Jones et al., 1997). There also is a difference in cytochrome oxidase activity in the posterior anterior hypothalamus, an area replete with neurons containing GnRH, which may partly explain the physiological differences between 2M and 2F females.

It is clear that the intrauterine position, affecting early exposure to sex steroids, leads to differences within the same sex. The functional consequences of these differences are as yet not clear. Experiments in semi-natural environments indicate that 2M females have larger home ranges (Zielinski et al., 1992), suggesting that they might do better in high density. There is some evidence that mammalian mothers can vary the sex ratio of their litters, even between the two different uterus horns (Clark et al., 1991b) and by doing so they will influence the frequency of 2M females. This opens the possibility that females can adjust the frequency of 2M females according to the situation in which they are reproducing. Whether the effects of intrauterine position are adaptive or not needs further study.

Maternal hormonal condition during pregnancy—During pregnancy the hormone production of the mother varies, in relation to the nature and duration of the pregnancy, genetic factors, and stressful events. For example, in the Mongolian gerbil, circulating concentrations of androgens in the maternal circulation is correlated with the foetal sex ratio (Clark et al., 1991a), which in turn can influence the behaviour of the mother. In addition, steroid hormones from maternal origin such as androgens and corticosterone and cortisol can reach the foetus via the placenta. The placenta, itself an endocrine organ, can metabolise maternal steroid hormones, thereby protecting the foetus from maternal hormones, but can do so only to a limited extent. As a consequence the hormonal status of the mother during pregnancy can affect the development of the foetus. The most conspicuous example is the case of the spotted hyena, in which females are dominant over males and in which there is heavy sibling competition between the young pups. The clitoris of female young is greatly enlarged, forming a pseudo penis. It is thought that this masculinisation and/or early aggression between pups come in part from the high androgen levels females have during pregnancy (e.g., Licht et al., 1992; Goymann et al., 2001). Similarly, gender roles of girls in humans relate to levels of testosterone in the blood of their mothers when they were pregnant with these children (Hines et al., 2002).

Another well-studied example is the effect of early maternal stress. For example, handling, or treatment with stress hormones of pregnant dams, or exposing them to an unstable social environment will not only influence the behaviour and physiology of the mother but also of her young. This affects their later stress sensitivity, hypothalamo-pituitary-adrenal axis, social behaviour and cognitive functions (Weinstock, 1997), as well as the masculinisation of female pups (Kaiser et al., 2003). Part of these effects are probably directly due to the effect of the

elevated levels of stress hormone in the mother on the developing foetus, while another part can be attributed to changes in her early maternal behaviour. So far, most of the effects on the offspring are interpreted as pathological and detrimental. It is, however, conceivable that the Darwinian fitness of the offspring is maximised and adjusted via this maternal hormone exposure to the stressful conditions to which the mother is exposed and that will be the environmental condition of the offspring too. For example, pregnant mothers that experience frequently stressful encounters with predators may prepare their offspring to this situation by making them more cautious and fearful.

Effects of maternal androgens: the avian model—Mammalian species have several disadvantages for the study of the effects of maternal hormones on development. First, the relationship between blood levels of hormones in the mother and what actually reaches the embryo is difficult to quantify because the placenta acts as an inter-phase between both and metabolises hormones itself. Second, early exposure to maternal hormones fluctuates while, as we have seen, hormones from siblings can also reach the embryo. Finally, manipulation of exposure of the embryo to hormones, indispensable to test hypotheses of descriptive studies, is difficult since the embryo develops inside the mother's body. Fortunately, many animal species produce eggs that develop outside the mother's body. In many of such oviparous species, including fish, turtles, reptiles, and birds, these eggs contain substantial levels of maternal hormones. There are two reasons why birds are especially suitable for studying the patterns, causes and consequences of maternal hormone deposition in eggs. First, birds produce relatively large eggs, facilitating experimentation and the determination of hormone levels in individual eggs. This facilitates the causal analyses of the hormone mediated developmental processes. Second, the ecology of several bird species is well known. This facilitates the analyses of functional and evolutionary aspects of these processes.

It was mainly Hubert Schwabl who opened up this field of research with the discovery that canary eggs contain androgens that affect the growth of the offspring from these eggs (Schwabl, 1993). Since then the field has rapidly expanded. So far, all bird species of which their eggs have been analysed on steroid hormones appear to produce eggs that contain substantial levels of androgens: androstenedione, testosterone and dihydrotestosterone. Furthermore, the levels of these androgens fluctuate systematically both within the clutch and between clutches. We will discuss both in this order.

i) Within clutch variation: Most of the attention in the literature has so far been given to within clutch variation. The majority of bird species produce clutches of several eggs that are laid with at least a time interval of one day. In several of these species levels of maternal androgens increase with the order in which the eggs of the clutch are laid. For example, in black-headed gulls (*Larus ridibundus*), androgen levels strongly increase with the laying order of the three eggs of the clutch (fig. 4). It has been hypothesised that such patterns are adaptive since the mother can compensate in this way for the disadvantage of the asynchrony in hatching between the different chicks (Schwabl, 1993). For example, in black-headed gulls, incubation starts when the first egg is laid, so before clutch completion. As a consequence, the first egg will hatch before the second and the second before the third. This will give chicks of later-laid eggs, that are younger and weaker than their older siblings, a disadvantage in sibling competition, since they completely rely on food from the parents, for which they have to compete. This increase in androgen deposition to last-laid eggs may mitigate the disadvantage of hatching asynchrony since it may enhance the competitive ability of last-hatched chicks. Indeed, canary chicks that hatched from eggs injected with androgens begged more frequently and grew more rapidly than chicks from control eggs (Schwabl, 1996). However, these effects were apparent only the first days after hatching and obtained under artificial conditions. Therefore the Groothuis laboratory set out to do a similar experiment in the field. First-laid eggs collected in black-headed gull colonies were found to contain relatively low levels of

androgens (see above). Next, clutches of three first-laid eggs were composed and cross-fostered to other gull pairs. Each set of three eggs was selected such that the eggs would hatch about one day after each other, as in natural clutches. In experimental clutches, the egg that would hatch first was injected with vehicle only, the second with a low dose of androgens and the one that would hatch last with a higher dose of androgens. In control clutches, all three eggs were injected with vehicle. In this way experimental nests were created that mimicked the natural situation (fig. 4) and 'control' clutches that lacked this pattern of androgens (fig. 5c). Time of hatching and growth up to the fledging stage was measured. The interval between hatching of the first and the third egg was smaller in the experimental clutches, indicating that yolk androgens shorten the degree of hatching asynchrony. Further, chicks of third eggs of experimental clutches grew faster than of third control eggs (fig. 5b). Both results indicate that elevated levels of maternal androgens in the egg may indeed partly compensate for hatching asynchrony. Interestingly, chicks from first eggs of experimental clutches (HO eggs, fig. 5c) that had to compete with siblings treated with hormone (H1 and H2 eggs) did not grow as fast as first hatched chicks from control clutches (OIL A) that had siblings that received vehicle only (fig. 5a). This indicates that first chicks in experimental clutches suffered from increased competition with the siblings that had hatched from eggs treated with androgens, suggesting that yolk androgens indeed affect competitiveness (Eising et al., 2001).

The latter interpretation was supported by another field study. In this instance the Groothuis laboratory composed clutches of two first-laid eggs, matched for weight and hatching time. One was injected with androgens, the other with vehicle. After hatching, the behaviour of the chicks was recorded at the times the parents came in to feed the chicks. Chicks of androgen-treated eggs were more alert, reacted more often as the first one to the parent, begged more, and obtained a larger share of food. This indicates that the effect of yolk androgens on growth is mediated by the effect of the androgens on behaviour (Eising et al., 2003). These data fit the finding (Lipar et al., 2000) that yolk androgens enhance the development of the neck muscle in the very young chick, since this muscle is important both for begging (a conspicuous pumping movement of the neck and head) and for hatching behaviour. In the same experiment early survival was enhanced in chicks from androgen-treated eggs, indicating a beneficial effect of maternal androgens on an important fitness parameter.

Although these data support the hypothesis that the pattern of androgen deposition over the eggs of a clutch is adaptive, they do not allow the conclusion that individual mothers are able to adjust this allocation pattern to the actual level of hatching asynchrony that shows considerable variation among and within species: A higher degree of hatching asynchrony should require a stronger increase of hormone levels over the laying sequence than a smaller degree. The problem of disposition adjustment has been approached at the level of between species comparison. For example, in some species that show hardly any hatching asynchrony, changes in androgens over the laying sequence are less strong. Moreover, in a siblicidal species, in which the oldest sibling, when it hatches healthy, kills off the younger one, to adjust the brood size to food availability, mothers deposit more androgens in first-laid eggs perhaps to facilitate the process of siblicide (Schwabl and Mock, 1997). However, this does not tell us whether within species individual mothers are flexible in their deposition strategy.

The Groothuis laboratory has tried to answer this question by looking at variation in hormone allocation within the same species, again the black-headed gull. To this end full clutches were collected in the field just after the last egg was laid. Eggs were then incubated for a standard time, after which the size of the embryo was measured and yolk hormone levels determined. Embryo size of the first-laid eggs was always larger than that of the last-laid egg because it had already been incubated for some time in the field. Based on an independent data set of eggs incubated for various periods of time, the length of time the first egg had been incubated before incubation on the third egg started could be estimated on the basis of embryo size. This

in turn gave an indication of the expected degree of hatching asynchrony. This estimate positively correlated with the increase of androgens over the laying sequence. In other words, clutches in which hatching asynchrony was expected to be large also showed large differences in androgen content between the third and first egg. The degree of hatching asynchrony is dependent on the incubation pattern. Incubation in birds is under the control of prolactin that at the same time influences androgen production. Therefore these data not only provided evidence for individual adjustment of maternal hormone allocation, but also a mechanism underlying this (Müller et al., in press).

However, the relation between hatching asynchrony and androgen deposition in the egg is probably more complex (Groothuis et al., 2005). Whether mitigating the effect of hatching asynchrony is adaptive depends on the function of hatching asynchrony itself, for which many hypotheses have been put forward, and on the extent to which avian mothers are able or constrained to manipulate the degree of hatching asynchrony. For example, in open ground breeders such as gulls, parents may have to sit already on their first egg when egg predation is high or because weather conditions such as sun radiation may impose detrimental effects on egg viability. Differential androgen deposition may then be the only option to counteract the by-product of early incubation on hatching asynchrony. In addition, in species in which siblicide only occurs under poor food conditions such that parents cannot rear the full brood, the androgen deposition pattern may depend strongly on the current food situation.

ii) Between clutch variation: Adjustment to hatching asynchrony cannot be the sole explanation for variation in maternal androgen allocation to the eggs, since there is also considerable variation in androgen levels between clutches (reviewed in Groothuis et al., 2005). Further, all eggs, and not only those late in the laying sequence, contain these hormones (fig. 4). At least part of this between-clutch variation is related to the social environment of the mother. It is well known for many animal species, including birds, that the production of gonadal steroid hormones is influenced by social factors such as the presence and quality of the other sex and the level of social competition. Such factors may stimulate androgen production in the female around the time most of the yolk of the eggs is deposited, a few days before ovulation and egg laying. Hormone levels in the egg may be a reflection of the hormonal state of the mother, induced by the social situation. We have found some evidence for this. Black-headed gull pairs that have larger territories and are most likely more aggressive (aggression being under the influence of androgens) produce clutches with relatively high levels of maternal androgens (Groothuis et al., 2002). The same was found for pairs nesting in places for which it is likely that a high level of competition exists (Groothuis et al., 2002). Evidence from other studies with other bird species is consistent with this (reviewed in Groothuis et al., 2002). In addition Gil et al., (1999) found enhanced levels of androgens in eggs of females paired with attractive mates compared to those paired with unattractive mates, a finding recently replicated in a more natural set-up (Von Engelhardt et al., unpubl.).

The question can be raised whether the relation between the social environment of the mother and hormone levels in her egg is just an epiphenomenon or an adaptation. Given the strong effects of maternal androgens on development we believe the latter to be the case. By providing her broods with more androgens when the mother experiences a higher level of competition, she may prepare her offspring for the higher level of competition they may encounter after hatching. By doing the same when paired with an attractive male, she may invest more in offspring from a high quality father (assuming that higher deposition requires higher circulating levels of androgens in the female that may impose some costs to her, cf., Clotfelter et al., 2004). However, the first explanation cannot apply for altricial species in which brood competition only occurs after fledging. This suggests that exposure to maternal hormones in the egg may have long-term consequences for the phenotype of the offspring, either because it adjusts the phenotype to the level of competition that the individual may encounter during

reproduction in the natal colony, or because the production of different phenotypes is in itself advantageous.

iii) Long-lasting effects: We have seen that exposure to elevated levels of maternal androgens in the egg can enhance growth and thereby fledging weight (Eising et al., 2001). It has been demonstrated in several bird species that fledging weight determines survival in first winter, and also later social status and reproductive success. In this way maternal androgens can have long-lasting effects on the offspring. In addition, these hormones may lead to individual differentiation in a more direct way. It is well known that early exposure to androgens can have organising effects on brain and behaviour. Indeed, manipulation of androgen and oestrogen levels in bird eggs is well known to affect sexual differentiation. However, by manipulating levels of androgens in the eggs within the range of natural within-clutch differences, we applied levels that were much lower than those used in studies on sexual differentiation. Still, these manipulated levels affected begging behaviour up to the age of 4 weeks (Eising et al., 2003). Moreover, in house sparrows it was found that androgen levels in the yolk correlated positively with social rank order among juvenile birds hatched from these eggs (Schwabl, 1996). However, this was a correlational study and since yolk levels of androgens can correlate with other aspects of the egg (Groothuis et al., 2002), an experimental approach is required to answer this question. To this end data on social behaviour and nuptial plumage of black-headed gulls were collected 10 months after they had hatched from either eggs injected with androgens or vehicle only. The data provide strong evidence for long lasting effects of maternal hormones (Eising, 2004). This suggests that by providing her broods with more androgens in areas of high competition, the mother translates environmental conditions, affecting her own hormone production, to the next generation.

EXPERIENCES DURING INFANCY AND IN ADULTHOOD

So far we have emphasised the important role of prenatal environmental factors on the development of social behaviour. However, this is not the only important life history phase for the development of this behaviour. After hatching or being born, the animal starts to interact with its social environment. Numerous studies have elucidated the role of social experience in shaping social behaviour. We will illustrate the influence of some specific social factors in leopard geckos, mice and birds, respectively.

Social experience in the leopard gecko

Experience during adulthood can also influence brain neurochemistry and behaviour in leopard geckos. It is well known that, relative to sexually naïve males, sexually experienced male rats and cats initiate copulation sooner, tend to be more aggressive, continue to copulate longer after castration, and respond more rapidly to androgen replacement. It is also common to find in mammals that experienced males exhibit greater changes in sex steroid hormone concentrations and immediate early gene expression when presented with cues that predict the introduction of a female. Altogether, it appears that sexually experienced males are more primed for sexual behaviour.

Similar effects of sexual experience are found in the leopard gecko. For example, sexually experienced male geckos begin to mark sooner, are less likely to flee from a territorial male, and have higher circulating concentrations of testosterone than naïve males (Crews et al., 1998; Sakata et al., 2002). In general we find that sociosexual experience also increases metabolic capacity in certain nuclei but reduces it in others; there also are nuclei where there is no discernable effect. However, this effect of sexual experience on both volume and metabolic capacity of brain nuclei is dependent upon incubation temperature. Again, the effects can vary from brain area to brain area. For example, the volume of the preoptic area increases with sexual experience in low-temperature females, but not in females from the male-biased

incubation temperature, whereas cytochrome oxidase activity in the ventromedial hypothalamus increases in females from the male-biased incubation temperature, but not in low-temperature females.

Another wrinkle comes in when we consider age. We have looked at this question by incubating eggs at different temperatures, raising hatchlings in isolation before transferring them to breeding cages on their first birthday or several years later. In leopard gecko life this corresponds to comparing an 18-year-old human with a 36-year-old. In this way we are able to assess the relative effects of age independent of social and sexual experience and, further, to determine if embryonic experience could affect the response.

As mentioned above, sexual experience can increase the volume of the preoptic area in females, whereas age can decrease the volume of the preoptic area. This points to an important principle that often is not taken into account in psychobiology or in phenotypic plasticity studies, namely that organisms age as they gain experience, but do not necessarily gain experience as they age. The only other example that we have been able to find that controlled for experience independent of age was a study of Witkin (1992) demonstrating that aging in rats is associated with a decline in the density of synaptic input to GnRH neurons in the preoptic area, but that reproductive experience will counter this trend and maintain synaptic input in old females at the levels of young adults.

Finally, the Crews laboratory has discovered that sexual experience can reorganise the functional associations between brain nuclei. Jon Sakata has developed methods to analyse correlations between cytochrome oxidase levels in different brain nuclei and combining these results with knowledge of neuroanatomical pathways (Sakata et al., 2000). Such analysis shows that, among some nuclei, sexual experience has no influence on the strength of neural connections but, among other nuclei, the functional associations are altered completely.

Taken together, such results indicate; i) that the volume and metabolic capacity of specific brain regions are dynamic in adulthood, changing as individuals age and gain sociosexual experience; ii) that the size and activity of brain areas can be independent; and iii) the embryonic environment influences the nature and degree of these changes.

Social experience in mice

In mammals it is common in these types of studies for the sex ratio of the litter to be balanced such that there are equal numbers of male and females and to contrast them with single sex litters; but in nature sex ratios in the litter vary.

This is particularly important when considering that the behavioural phenotype of knockout mice is often interpreted as the effects of the absence of the gene product on adult behaviour. Could these behavioural differences among genotypes be exaggerated or blurred by the postnatal environment? That is, since mice develop in litters of varying sex ratios and genotypes, it is possible that some of these behavioural differences may result from the unique composition of the litter. To determine if these factors might play a role in the development of the behavioural characteristics that have become diagnostic of knockout mice, Crews et al. (2004) sexed and genotyped within 2 days of birth pups derived from mating of males and females heterozygous (HTZ) for a null mutation of oestrogen receptor α (ERKO). Litters were then reconstituted, forming same-sex/mixed-genotype litters of equal numbers of ERKO and wildtype (WT) individuals. In this manner any effect of sex could be dissociated from any effect of genotype. As adults, ERKO and WT individuals were tested in a standard resident-intruder paradigm. The results indicated that the behavioural differences between the genotypes were more sharply defined than reported previously. ERKO females displayed only aggressive behaviour whereas their WT litter mates displayed only mounting behaviour; in ERKO males

both aggression and mounting behaviour was greatly reduced (fig. 6). These data suggest that the postnatal environment such as litter composition may influence the development of sociosexual behaviour.

Social experience in avian species

As we have seen above, mothers can adjust the competitiveness of their offspring by providing them with embryonic exposure to her androgens. In addition, she may influence competitive behaviour of her young by providing them with a certain social environment after hatching. The effect of social experience comes about partly in interaction with hormonal factors. Social factors may stimulate hormone production, influencing the performance of social behaviour, while hormones may bring the animal into a situation in which it is able to gain social experience. This interaction may influence the form, application and frequency of social behaviour. We will discuss these three aspects briefly in this order.

The motor coordination for the basic form of species-specific postural and vocal displays in gulls and other non-oscine avian species is already present early in ontogeny, and can be activated precociously by early testosterone treatment even without substantial social experience or practice (Groothuis et al., 1992). Nevertheless, details of these motor patterns such as the position of the bill or wings can be shaped by social experience, probably due to operant conditioning, based on the effect a display has on its opponent (Groothuis, 1992). This demonstrates that an environmental factor that is not indispensable for normal development can still affect development when present. This shows the invalidity of 'kaspar hauser' experiments, often used at the time of Tinbergen and the nature-nurture debate. Such experiments, in which animals are reared in social isolation, can only tell us which factors are not indispensable for normal development, but not how normal development takes place, let alone the influence of genetic factors that are not even manipulated in such an experiment.

However, such isolation experiments have shown that social experience is indispensable for the development of the proper use of threat and courtship displays. Unfortunately, complete social isolation is such a crude manipulation that the cause of abnormal behaviour is difficult to interpret. Groothuis refined this approach, and reared gull chicks in small sibling groups. Since in such groups hardly any aggressive interactions take place, Groothuis could specifically deprive the young from having such interactions without refraining them from other social input. Such birds did not show the gradual shift from overt aggression to the use of threat display that normally-reared birds show during their first year. This indicates that birds have to gain experience with social interactions to develop efficient communication behaviour (Groothuis, 1992, 1993).

Finally, the frequency of social behaviour is obviously dependent upon the number of social interactions, which in turn stimulate androgen production and facilitate social behaviour. A central framework for the social regulation of androgen production is the challenge hypothesis (Wingfield et al., 1990). Assuming that testosterone imposes costs to the animal, circulating levels of this hormone should only be elevated during or in anticipation of a social challenge. The laboratory of Groothuis recently demonstrated for the first time that this is the case in young birds too (Ros et al., 2002). Black-headed gull chicks that had to defend their territory more frequently had elevated levels of the hormone, and also produced short-lasting peaks of testosterone contingent on a social challenge (an artificial intruder at the territory). Interestingly, birds that experienced elevated levels of testosterone in the second week after hatching became increasingly sensitive to the hormone and this effect lasted at least until fledging. This demonstrates, in addition to the effect of embryonic exposure discussed above, another long-lasting effect of early testosterone exposure. Thus, in black-headed gulls, mothers can influence the level of social stimulation her chicks receive, and thereby their early exposure

to testosterone, by her choice of nest site. Chicks reared in higher densities will experience higher levels of social stimulation and thereby higher levels of androgens (Ros, 1999).

CONCLUSION

We hope to have demonstrated that within the field of ethology the question of behavioural development is still a question in its own right. Of course, the field has progressed enormously since Tinbergen, and has made the nurture-nature debate obsolete. The data presented in this paper emphasise how subtle influences from the embryonic environment strongly influence species-typical behaviour and are often mediated by maternal effects. Clearly, research in the area of behavioural development has left the stage of black-box analyses and now deals with the level of physiological processes and gene expression. In addition we are now much more aware that such research should include both proximate and ultimate approaches. Finally, we hope to have convinced the reader that we should now not only focus on the developmental pathways for species-typical behaviour, but also try to explain variation within the same species and sex.

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Figure 1.

Incubation temperature results in different sex ratios in the leopard gecko (*Eublepharis macularius*) as well as multiple phenotypes within each sex. Panel A indicates how only females result from a 26°C incubation temperature (white) while at other temperatures both sexes are produced in different sex ratios. 30°C females are shown in light grey, 32.5°C females are shown in medium grey, and 34°C females are shown in dark grey. Males are produced at three of these incubation temperatures: 30°C males are shown in medium grey, 32.5°C males are shown in dark grey, and 34°C males are shown in black. Panel B indicates how within each sex (females top panel, males bottom panel) all morphological, physiological, neural, and behavioural traits measured to date differ according to incubation temperature, yet when combined across temperatures, are normally distributed.

Figure 2.

Incubation temperature influences mate choice behaviour in adult male leopard geckos (*Eublepharis macularius*). Illustrated are mean and standard deviation of the time spent in compartment before female chamber. In Panel A, males (light grey) from a 30°C incubation temperature (which produces a female-biased sex ratio) spend significantly more time in front of the compartment containing a female from a 34°C incubation temperature relative to the compartment containing a female from a 30°C incubation temperature. On the other hand, in Panel B we see that males (dark grey) from 32.5°C incubation temperature (which produces a male-biased sex ratio) spend significantly more time in front of the compartment containing a female from a 30°C incubation temperature relative to the compartment containing a female from a 34°C incubation temperature.

Figure 3.

Metabolic capacity in the interconnected limbic nuclei of adult male leopard geckos from 32.5°C incubation temperatures (top panel) and from 30°C incubation temperature (middle panel). Bottom panel indicates the difference between the neural landscapes, revealing the effect of temperature during incubation on metabolic activity in the adult brain. Illustrated in least squared means of cytochrome oxidase (COX) activity. All nuclei accumulate sex steroid hormones and are involved in the expression of social behaviours. Positive peaks indicate nuclei in which change was more positive in males from a 32.5°C incubation temperature, whereas negative peaks indicate nuclei in which change was more positive in males from a 30°C incubation temperature. Asterisk indicates significant difference. AH (anterior

hypothalamus), VMH (ventromedial hypothalamus), AME (external amygdala), SEP (septum), PP (periventricular nucleus of the preoptic area), POA (preoptic area), NS (nucleus sphericus).

Figure 4. Levels of androgens (androstenedione plus testosterone) in the yolk of freshly laid eggs of the black-headed gull in relation to the position of the egg in the laying order. Lines connect clutches. $**p < 0.01$.

Figure 5.

Body mass changes with age of chicks hatching from eggs treated with different doses of a combination of androstenedione and testosterone. Plotted are the differences with body mass of chicks that hatched from B eggs in oil clutches. A: First-hatched chicks of experimental clutches (hormone clutches in which eggs were treated with increasing levels of androgens: H0 = oil only, H2 is highest dose of androgens) grow less well than first-hatched chicks of control nests (all eggs injected with oil only), due to increased competition by siblings of H1 and H2 eggs; B: Third-hatched chicks from experimental eggs, receiving the high dose of androgens, grow better than third-hatched chicks of control eggs; C: experimental design.

FEMALES **MALES** **Figure 6.**

Frequency of aggressive and mounting behaviour in genetically manipulated mice raised in single sex groups with mixed genotypes. Tests with female mice involved ovariectomised female intruders (top panel) and with male mice involved olfactory-bulbectomised male intruders (bottom panel). Mice were genotyped within 2 days of birth and the litters reconstituted to contain equal numbers of wildtype (WT) or knockout (KO) male or female mice. Values are group mean and standard errors. Statistical analysis was computed on log-transformed data; one-tailed t tests (* $p < 0.05$, ** $p < 0.01$). (From Crews et al., 2004.)