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From gene networks underlying sex determination and gonadal differentiation to the development of neural networks regulating sociosexual behavior

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ABSTRACT

Genes are not expressed in isolation any more than social behavior has meaning outside of society. Both are in dynamic flux with the immediate environment that the gene/individual finds itself, which in turn establishes the timing, pattern, and conditions of expression. This means that complex behaviors and their genetic underpinnings should be viewed as a cumulative process, or as the result of experiences up to that point in time and, at the same time, as setting the stage for what will follow. The evidence indicates that as experiences accumulate throughout life, early experiences shape how genes/individuals will respond to later experiences, whereas later experiences modify the effects of these earlier experiences. A method of graphically representing and analyzing change in gene and neural networks is presented. Results from several animal model systems will be described to illustrate these methods. First, we will consider the phenomenon of temperature-dependent sex determination in reptiles. We will illustrate how the experience of a particular temperature during a sensitive period of embryogenesis sculpts not only the patterns of expression of genes involved in sex determination and gonadal differentiation but also the morphological, physiological, neuroendocrine, and behavioral traits of the adult phenotype. The second model system concerns the effects of the sex ratio in the litter in rats, and the genotype ratio in the litter of transgenic mice, on the nature and frequency of maternal care and how this in turn influences the patterns of activation of identified neural circuits subserving the offspring's sociosexual behavior when it is an adult.

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"The interaction out of which the organism develops is not one, as is so often said, between heredity and environment. It is between organism *and environment! And the organism is different at each stage of its development."* (p. 345; emphasis in the original)

Daniel S. Lehrman (1953)

"The nervous organization on which response to sexual stimulation depends is influenced at very early age by contact with other animals…" (p. 1219)

William C. Young (1961)

1. Introduction

The above quotes impress upon us that at any point in time all traits, whether it is an organism's behavior, its physiology, or the patterns of gene expression during the formation of a tissue, have a history that has shaped its immediate expression. This history can be on a scale of seconds and minutes, an individual's lifetime, over generations and ultimately through evolutionary time. The challenge then is how to study this constant yet ever changing complexity.

Most scientists take the strategy of changing single variables and observing the outcome, assuming incorrectly that the observed result is due to the manipulation and the measured variable alone. Although it has long been appreciated that the product(s) result from the dynamic and reciprocal exchange between the trait and its external environment and internal milieu, efforts to study the process have usually been identified with individual scientists rather than to schools of thought. This minority approach of casting the net wide to determine how change affects the web of causative elements has had a disproportionate impact on scientific advances.

We offer here several case studies from our recent work in developmental genetics and developmental neuroscience to illustrate one such attempt. Rather than incorporating many variables, we limited our experiments to a handful. As will be evident, even this very focused strategy reveals emergent properties within systems that have not been sufficiently appreciated. The phenomena of environmental regulation of genetic cascades in temperature-dependent sex determination (TSD) and the role of experience in shaping the neural substrates that underlie sociosexual behavior in adult reptiles and mammals serve as particularly good examples of how this interaction modifies the adult phenotype. In both epigenetic factors (meaning outside the gene) play a fundamental role in how the individual develops in the way that it does. We will see that although the triggers of sex determination may differ, the mechanisms and processes that lead to a functional male or female are similar. Alternatively, we will see how similarities in behavioral expression can be underpinned by very different patterns of neural activity.

2. Genetic cascades and neural networks

Complex traits are not particularly susceptible to conventional analysis. A first order strategy unraveling this complexity has been to single out a particular trait(s), in this instance genes and brain nuclei and their respective roles in sex determination and sociosexual behavior, and apply any of an increasing variety of sophisticated methods to study their function. Although very successful in its own right, this approach has created problems for understanding the developmental and environmental contributions to complex traits. That is, they tend to ignore the context, whether it is the timing, patterning, or the conditions in which the trait manifests and progresses. Fortunately, more researchers are coming to appreciate the interrelated nature of traits at all levels of biological organization and, in so doing, have begun to look at the interactions of traits rather than considering them as independent variables.

With the development of techniques such as DNA microarray in which thousands of genes can be studied simultaneously and the bioinformatic tools enabling truly comparative genomics, we are seeing major advances and fuller understanding of complex traits (e.g., the human diseases leukemia and chronic fatigue syndrome). Both of these research efforts have identified remarkably few genes that form unique fingerprints reflecting predispositions in responsiveness to stimuli peculiar to life history stages, environmental, and/or physiological contexts.

In the area of behavioral neuroscience, Newman (1999) made a strong case that the days of studying a single or even a few nuclei in relation to sociosexual behavior are over. Rather, specific limbic nuclei are reciprocally interconnected and define circuits that are sensitive to both somatosensory and hormonal stimuli. Newman proposed a hypothetical three-dimensional representation of how the correlated data might be compiled to allow one to see how the activity of the nuclei in the network may differ in different behavioral states.

However, even when the multiple components of complex traits are examined as a unit, such as the suite of genes known to be involved in sex determination and gonadal differentiation or the neural circuitry underlying sociosexual behavior, conventional analytic and presentation methods make it difficult to quantify and illustrate the information.

A previous paper has addressed alternative qualitative and quantitative methods that are particularly useful in analyzing behavioral and physiological data (Fuller et al., 2005). The present paper focuses on a new method of presentation of systems analysis that avoids the problems inherent in dense tables or complex graphics. This method not only visualizes the constituent elements of complex traits and their interaction but calculates the effects of time and experience on these systems.

This method can be viewed as a recent addition to the long history of imagery to depict complex concepts in all areas of science. Well-known images in biology would include Waddington's (1957) developmental landscape depicting the genes that shape tissues and, more recently, Nijhout's (2003) schematic of the importance of context in trait development. Similarly, in psychology, there is Gottesman's (1997) depiction of the contribution of genes to cognitive ability and that of Grossman et al. (2003) illustrating how genetic and experiential factors push the individual to thresholds of pathology. Notably, all share the use of three dimensions to illustrate

complex traits whose individual components are two-dimensional in nature.

3. The functional landscape method

Any method should have certain attributes. Principally, it should accommodate the possibility that the different components may have different scales of measurement, enable the measurement of the network of interrelated components as a whole, and how it changes with time or manipulation. A new method is illustrated here that uses both published and unpublished data derived from experiments on reptiles and mammals.

The steps of this analytic procedure are as follows.

First, the elements are selected on the basis of empirically established associations. These can be group means of the abundance of a particular gene product(s). By plotting these values in three dimensions using a MATLAB program (The Mathworks), a landscape is produced in which actual values are at the apex or nadir of each mountain or valley, respectively. It is important to understand that the slopes of these peaks and valleys have no empirical meaning in this method, but are a function of the number of elements measured; the more values plotted, the closer each is to one another and, depending upon the scale, the steeper the slope. This method is optimal for fewer than ten genes or brain nuclei. Thus, it is excellent for depicting the relationship between genes and nuclei that have established functional relationships or within subsets of genes within a system and nuclei within a brain region. Finally, because the functional landscape maps are three-dimensional, peaks and valleys above and below the plane signify directionality of change.

Second, for integrative studies it would be an advantage to correlate different measurements (e.g., gene expression, physiological levels of hormones, metabolic activity in brain nuclei, and different behaviors). However, often the scales of measurement are very different for each component. For example, A may span $[0.10 \text{ to } 0.40]^{-5}$ whereas B may span 0.10 to 0.25. How does one accommodate different measures having different scales? This can be done using the percent (%) maximum method where the value of each element is normalized to all other values of that trait within groups being compared (Table 1). For example, a gene product may be expressed in very different amounts through time or have a different abundance compared to other genes at the same point in time. A standard method is to normalize according to the abundance of a housekeeping gene. This can then be followed by combining the values for the particular element from the groups to be compared, giving the highest value the maximum of 100%, and then expressing each value as a % of this maximum.

Third, because we are interested in changes in systems through temporal, spatial, and/or contextual conditions, it also is desirable to be able to illustrate these changes. This entails not only calculating differences between mean responses using conventional parametric statistics such as multivariate analysis of variance (MANOVA), but also utilizing the exhibited correlations via statistical methods including the generalized estimating equations (GEE) approach and the mixed effects models (e.g., Liang and Zeger, 1986; Lindstrom and Bates, 1988; Fitzmaurice et al., 2004).

Table 1 – Method for calculating percent maximum

Incubation temperature	Embryonic stage	Relative abundance	% Maximum
MPT	Stage 17	0.013	10
	Stage 19	0.078	63
	Stage 21	0.041	34
	Stage 23	0.120	100
FPT	Stage 17	0.015	12
	Stage 19	0.031	26
	Stage 21	0.003	3
	Stage 23	0.004	4

Above is the relative abundance of the gene *Sox9* in the gonad of red-eared slider turtle embryos during the period of sex determination (Stage 17) and gonadal differentiation (Stages 19, 21, and 23) while incubating at a male-producing temperature (MPT) and at a female-producing temperature (FPT). Abundance of mRNA determined using quantitative real-time PCR and expressed relative to the abundance of the housekeeping gene PP1. In this instance the highest value is at stage 23 at the MPT. Each value at other embryonic stages at the MPT as well as the FPT are then expressed as a % of this maximum.

The GEE and the mixed model approaches are generalizations of the standard linear model to allow for correlations and nonconstant variabilities inherent in the data. For example, in our data the individuals possessing the traits are independent, but multiple trait measurements taken from the same individual are correlated to some degree. The methods for analyzing correlated data provide the flexibility to model both the means and their variances and covariances for the purpose of drawing valid statistical inferences. In our analyses, the intra-subject correlations among multiple measurements are modeled via a range of covariance structures, including compound symmetry, Toeplitz, AR(1), unstructured, and spatial power. The parameters of interest are estimated using the method of restricted maximum likelihood. Akaike's and Schwarz's criteria are then used to compare different models (Akaike, 1974; Keselman et al., 1999; Schwarz, 1978).

4. Case study: sex determination and gonadal differentiation

Vertebrates exhibit two forms of sex determination, genotypic sex determination (GSD), and environmental sex determination (ESD). The most thoroughly studied is GSD, a process in which the sex of the individual is established at fertilization with the union of the male and female gametes and the inheritance of a specific gene(s) from one of the parents. In mammals this is *Sry*. Thereafter, a reliable and regular series of molecular events unfold that leads to the development of testes or ovaries, followed by the secretion of gonadal hormones that act throughout the body to shape the accessory and secondary sexual characters that characterize the male and the female. One form of ESD is temperature-

dependent sex determination (TSD). In this instance, each individual embryo is completely bipotential, capable of developing into a male or a female depending upon the temperature to which it was exposed in the middle third of incubation. Thus, whereas the trigger for determining sex, an inherited gene(s) in GSD vs. temperature in TSD, is fundamentally different, the functional binary outcome of male vs. female is the same (Crews, 1993).

Depending upon the species, the pattern of TSD may be one in which females are produced at low incubation temperatures relative to the temperatures that produce males; males produced at low temperatures relative to the temperatures that produce females; or females produced at temperatures at either extreme with males being produced at intermediate temperatures. It is notable that the transition from all-male to all-female-producing temperatures can be extremely sharp; in the red-eared slider turtle (*Trachemys scripta*) this transition occurs over a 0.8 °C range (Crews et al., 1994). Importantly, within temperature transition, the male–female ratio changes predictably rather than the hatchlings being hermaphroditic or intersex. In the leopard gecko (*Eublepharis macularius*) a different pattern of TSD is observed, with high and low incubation temperatures producing only females whereas intermediate incubation temperatures produce different sex ratios. That is, extreme temperatures (26 °C and 34 °C) are female-producing incubation temperatures, whereas intermediate temperatures result in different sex ratios: 30 °C (Tf) produces a female-biased sex ratio (25:75) and 32.5 °C (Tm) a male-biased sex ratio (75:25).

Finally, like in GSD species, sex determination and gonadal differentiation are distinct events in TSD (Fig. 1). In the slider turtle, the embryo becomes sensitive to temperature at embryonic Stages 15 and 17 and the gonad differentiates shortly thereafter, during Stages 19-23. This window of temperature sensitivity is a discrete period during which the sexual trajectory of the embryo can be manipulated by switching eggs from a male-producing temperature (MPT) to a female-producing temperature (FTP) (or vice versa) or by chemical manipulation (Crews, 1996; Crews et al., 1994, 2001).

In both modes of sex determination many of the same genes are involved in the differentiation of the gonad. Thus,

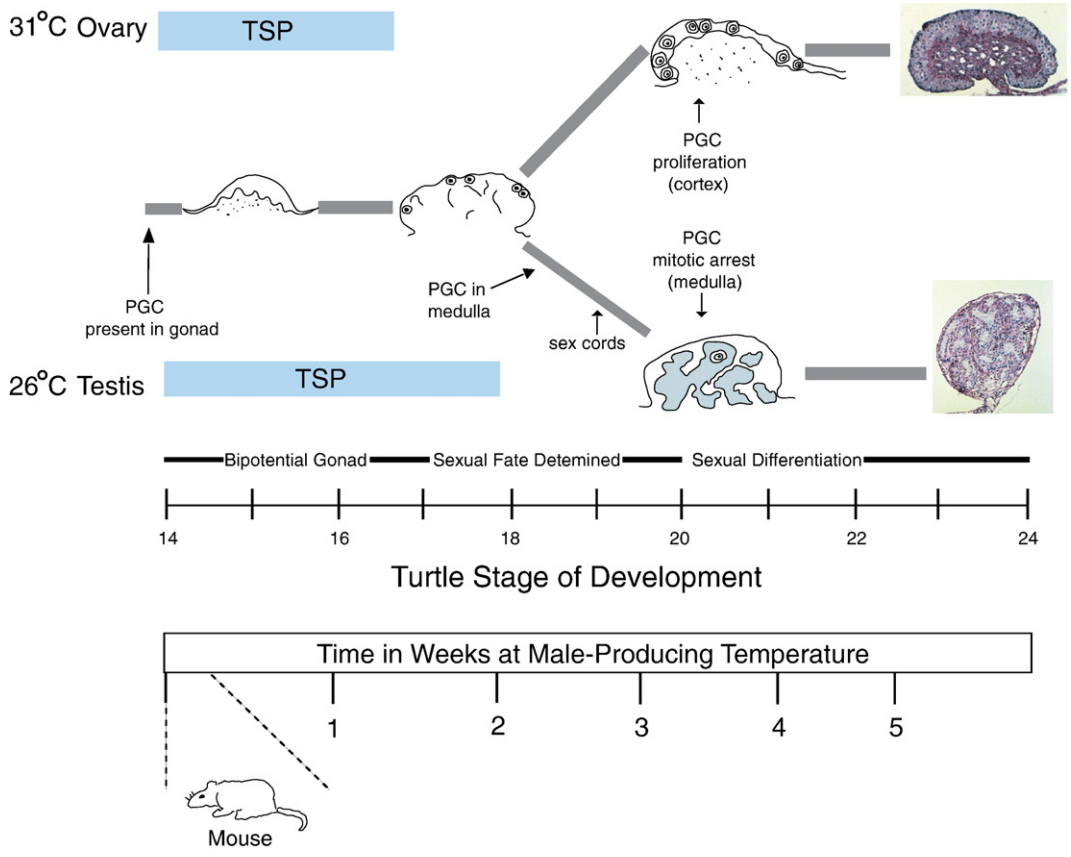

Fig. 1 – Incubation temperature during the temperature-sensitive period (TSP) determines the sex in the red-eared slider turtle (*T. scripta*). Gonad development can be divided into three periods: formation of the bipotential gonad, sex determination, and sexual (gonadal) differentiation. The gonad of turtle embryos is bipotential throughout the TSP, during which time incubation at 26 °C (male-producing temperature or MPT) leads to testis development and incubation at 31 °C (female-producing temperature or FPT) leads to ovary development. At MPT, primordial germ cells (PGCs) migrate into the medullary region (~embryonic stage 18), are enclosed in sex cords (~stage 19), and enter mitotic arrest. At FPT, PGCs proliferate in the rapidly growing cortex, shown at the top. Compared to the relatively short period of sex determination in mice (10.5 days), in *T. scripta*, this process may take up to 3.5 weeks in males and 1.5 weeks in females.

although upstream triggers, genetic or environmental, distinguish GSD from TSD, they both activate a conserved pathway of genes in regulating vertebrate gonadal differentiation. However, exactly how these transcription factors and signaling molecules interact in the context of the developing gonad remains, for the most part, unclear (Koopman, 2001). In our studies we have used comparative quantitative analysis of transcript levels within gonads of embryos developing at a FPT and at an MPT as well as whole mount *in situ* hybridization of specific molecules to identify their expression at a cellular level. Analyses of the patterns of gene expression at different embryonic stages allow first-order approximations of the relationships between genes in this network. It is convention to express measures of gene expression as proportional to a housekeeping gene. It is a mistake to assume that the housekeeping gene utilized does not change through time or state. Thus, the appropriateness of such measures should be established anew for each system under study.

We are investigating the involvement of seven genes (*Sox9*, *Mis*, *Dmrt1*, *Sf1*, *Wnt4*, *Dax1*, and *FoxL2*) in the process of sex determination gonadal differentiation in the slider turtle. These genes are also found in mammals where they are known to be involved in gonadal differentiation. For example, in mice, *Sox9* is expressed in pre-Sertoli cells following *Sry* expression and is required for *Mis* up-regulation (Koopman, 2001). *Sox9* is also expressed in a male-specific manner during the period of sex determination in birds and reptiles (Western et al., 1999, 2000; Torres-Maldonando et al., 2002; Oreal et al., 1998, 2002; Smith et al., 1999a). Taken together this suggests that *Sox9* plays a central and conserved role in vertebrate sex determination. The gene coding for Müllerian inhibiting substance (*Mis*) plays a conserved function as well and is both necessary and sufficient to cause Müllerian duct regression (Behringer et al., 1990, 1994). *Dmrt1* also is critical to sex determination, as its expression is detected in both Sertoli cells and PGCs (Raymond et al., 1998, 1999). Finally, the temporal and spatial expression patterns of *Sf1* in the gonad are conserved between turtle and mouse (Fleming and Crews, 2001; Fleming et al., 1999; Luo et al., 1994).

In comparison with testis development, little is known about the molecular genetics of vertebrate ovary differentiation, but several genes critical for the promotion of female development and suppression of the male pathway have been identified from studies of human genetic disorders. As in testis development, a number of these genes, including *Wnt4*, *Dax1*, and *FoxL2*, show similar patterns of expression during ovarian development in several vertebrate taxa (Smith et al., 1999b; Torres-Maldonando et al., 2002; Western et al., 2000; Loffler et al., 2003). This suggests that an ovarian pathway, complementary to the testis-determining pathway, is conserved among vertebrates. In both pathways these genes interact; for example, *Sf1* upregulates *Mis* whereas *Dax1* downregulates *Sf1*.

As indicated in Fig. 2, the abundance and pattern of change in the above mentioned genes during development differ according to incubation temperature in the red-eared slider. It is important to emphasize that all seven genes are expressed but in different amounts and at different times at both male- and female-producing incubation temperatures; the only exception appears to be *Mis*, which is active only at the MPT. Initially there is little difference, although *Dax1* and *Sf1* are expressed in greater amounts at the MPT. Toward the end of the sex determination period, the differences are more evident, with *Sox9*, *Dmrt1*, *Sf1*, and *Mis* being expressed at higher levels at the MPT. Interestingly, it is at this time that we observe *FoxL2* being produced in greater amounts at the FPT. As the gonads begin to differentiate, these differences continue, with the addition of *Wnt4* beginning to be expressed at higher levels at the FPT but *Dax1* beginning to be expressed at higher levels at the MPT. By the end of the period of gonadal differentiation, testes are marked by elevations in *Sox9*, *Dmrt1*, *Sf1*, and *Mis* whereas ovaries are characterized by continued higher expression of *FoxL2* and *Wnt4*.

5. Case studies: neural networks and the role of experience in their organization

How does experience modify the neural mechanisms underlying sociosexual behavior? In particular, how do experiences early in life and later in adulthood interact to affect adult sexual behavior and the underlying neural circuits? The proposition that embryonic experience interacts with adult experience in shaping behavioral phenotype is simple enough conceptually: adult animals change their behavior in response to experience, and the nature of this change depends on various factors, some of which depend on the embryonic and perinatal environment to which the animal was exposed. However, there have been relatively few studies that have undertaken this challenge. The Crews' laboratory has worked with three animal model systems, the leopard gecko, the rat, and the transgenic mouse in this regard; the research on the latter two systems have been in collaboration with Alison Fleming of the University of Toronto and Sonoko Ogawa of the University of Tsukuba, respectively.

For much of this work we have utilized the method of cytochrome oxidase (COX) histochemistry. COX is a rate-limiting enzyme in oxidative phosphorylation, the major pathway in brain metabolism, and consequently the abundance and activity of COX activity in a brain area is a measure of the metabolic capacity of that brain region. In other words, the COX abundance not only reflects the metabolic history of an area, but because it determines the amount of ATP available in a neuron, constrains the amount of activity a neuron can sustain (Sakata et al., 2005). Thus, COX is unlike 2-DG autoradiography or c-*fos* immunocytochemistry, which provide information on evoked or immediate activity, in that it reveals long-term changes in brain activity. Indeed, if the experience occurs early in development, this method can detect how metabolic activity has changed even several years after the event! We find that in both mammals and reptiles metabolic activity in limbic areas reflects the capacity to display sociosexual behaviors and, in turn, that differences in metabolic activity in these areas reflect individual differences in the propensity to display social behaviors.

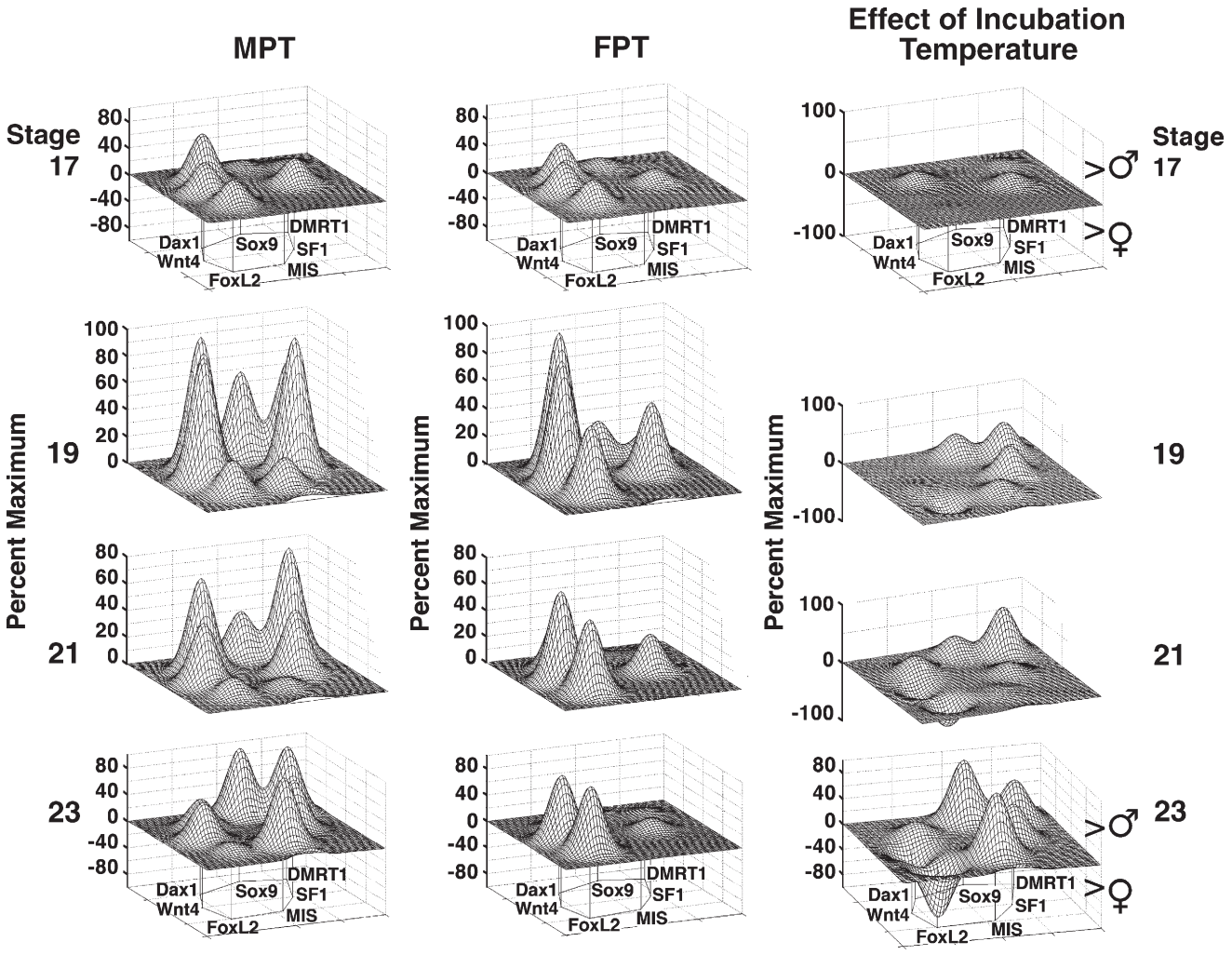

Fig. 2 – Although the triggers differ, the same genes are involved in the process of gonadal differentiation in species that exhibit genotypic sex determination (mammals and birds) and those that exhibit temperature-dependent sex determination. Illustrated is the abundance of various genes as measured by quantitative PCR during the periods of sex determination (embryonic Stage 17) and gonadal differentiation (Stages 19–23) of the red-eared slider turtle (*T. scripta*) embryos while incubating at a male-producing temperature (MPT) or a female-producing temperature (FPT) after standardization using the percent maximum method. Abbreviations: winged-helix/forkhead transcription factor (FoxL2); dosage-sensitive sex-reversal adrenal hypoplasia congenital critical region on the X chromosome (Dax1); DM-related transcription factor one (Dmrt1); Müllerian inhibiting substance (Mis); steroidogenic factor one (Sf1); SRY-related HMG box nine (Sox9); wingless-related integration site 4 (Wnt4). The symbols > ♂ and > ♀ indicate that expression levels are higher at the MPT or FPT, respectively.

5.1. *Incubation temperature defines the adult phenotype in the leopard gecko*

In TSD species the incubation temperature not only determines sex and the type of gonad that develops, it also shapes the entire adult phenotype. In this work we have used the leopard gecko (*Eublepharis macularius*), a solitary species. For these studies eggs were collected and incubated at a specified incubation temperatures, and the individuals followed throughout their life. Until sexual maturity the animals were housed in isolation but thereafter they were manipulated as adults in particular ways to identify the effects of incubation temperature, the effects of the manipulation, and the interaction between these early and later experiences.

An early finding, and one that continues to be observed as new traits are measured, is that incubation temperature not only establishes the gonadal sex of the individual, but also accounts for much of the within-sex variation observed in the morphology, growth, endocrine physiology, and aggressive and sexual behavior of the adult (Table 2) (reviewed in Crews et al., 1998; Sakata and Crews, 2004). For example, males in general grow more rapidly and are larger than females from the same incubation temperature; Tm males however grow more rapidly and to a larger size than do Tf males. From hatching to 2 and 10 weeks of age circulating concentrations of androgens (total androgens, or T and DHT assayed separately) are low in Tm and Tf males and do not differ; by 25 weeks of age, androgen levels increase but do

Table 2 – Incubation temperature during embryonic development not only determines the type of gonad that forms in the leopard gecko (*E. macularius*) but also shapes the adult phenotype

Trait	Male	Female
Growth	+	+
Sex Hormone Levels	+	+
Sensitivity to Sex Hormones	+	+
Sexual Behavior	+	+
Aggressive Behavior	+	+
Neurophenotype		
A. Preoptic area		
Metabolic activity	+	+
Volume	+	+
B. Ventromedial hypothalamus		
Metabolic activity	+	+
Volume	–	+
C. Amygdala		
Metabolic activity	–	+
D. Nucleus sphericus		
Metabolic activity	+	+

Depicted are different traits that are influenced by incubation temperature in gonadal males and females. Plus indicates significant effect. Minus indicates no effect. See reviews by Crews et al. (1998) and Sakata and Crews (2004).

not differ statistically (Rhen et al., 2005) or in adulthood. Estrogen levels do differ significantly, however, with Tf males having higher levels than do Tm males. Despite this similarity in circulating androgen levels in adulthood, males from the two temperature morphs differ significantly in their scent-marking response to exogenous hormones in adulthood, indicating neuroendocrine differences between the Tf and Tm males. There are also between-sex as well as within-sex differences in glucocorticoid levels in response to stress. As expected from work with other vertebrates, females have higher circulating levels of corticosterone levels than males, but, for both females and males, Tm individuals have significantly lower levels than did Tf individuals (Fig. 3). Brain neurochemistry is also influenced by incubation temperature. For example, a significantly higher number of TH-ir cells are found in the VTA of sexually inexperienced Tf vs. Tm males that had been castrated and androgen-implanted (Fig. 4A), suggesting that embryonic temperature does indeed play a role in differentially organizing dopaminergic systems of the temperature morphs. This is further supported by the finding of significantly higher DA levels were measured in the nucleus accumbens of Tf males compared to Tm male geckos that have interacted with a receptive female across a barrier (Fig. 4B). Finally, sexually experienced Tf and Tm males both show strong preferences in a Y-maze apparatus to females or their odors, but the type of female they choose depends upon their incubation history (Putz and Crews, 2006). Finally, among females, Tm females are less attractive to males than are Tf females and will even attack males, a typically male pattern of aggression.

Incubation temperature also influences the metabolic capacity of certain forebrain nuclei in adult leopard geckos, and further, these differences correlate with the differences exhibited in their sexual and agonistic behaviors as adults.

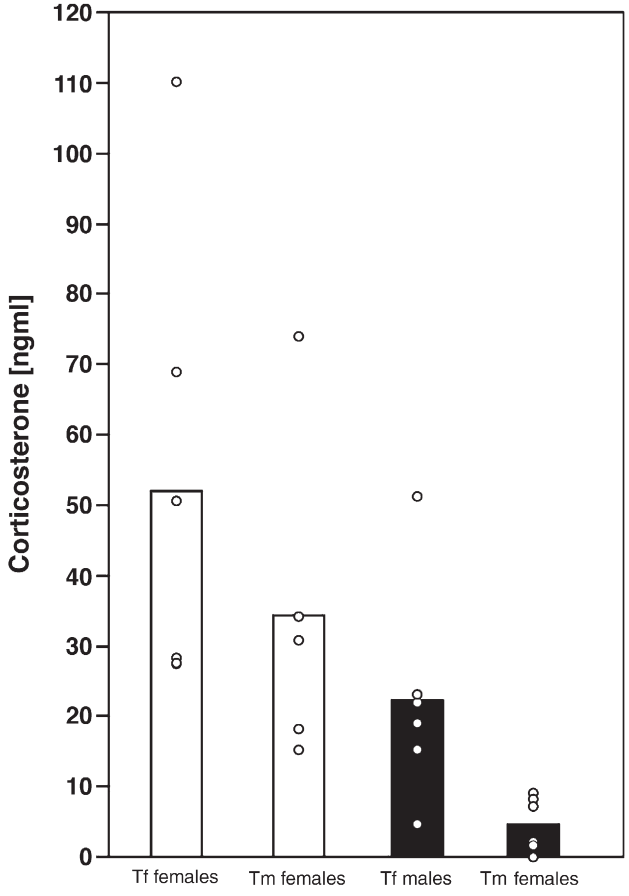

Fig. 3 – Corticosterone levels in male and female leopard geckos (*E. macularius*) from different incubation temperature after being placed in a brightly lit room, a mild stressor (Tf females vs. Tf males, $p<0.05$; Tm females vs. Tm males, $p<0.01$).

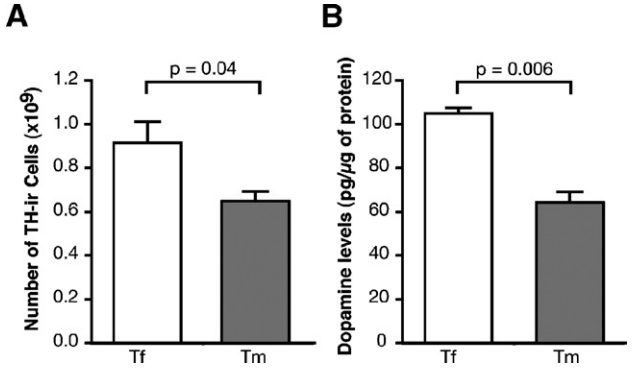

Fig. 4 – Incubation temperature influences neurotransmitter levels in sexually inexperienced male leopard geckos (*E. macularius*). Panel A: TH-ir cell counts are significantly different in the ventral tegmental area between castrated, androgen-implanted Tf and Tm male leopard geckos. Panel B: Dopamine levels in the nucleus accumbens of intact Tf and Tm male geckos exposed to a stimulus receptive female across a wire-mesh barrier.

For example, we have measured metabolic capacity in the septum, ventromedial hypothalamus (VMH), anterior hypothalamus, and nucleus sphericus (medial amygdala), preoptic area (POA), and the periventricular preoptic area (PP), all areas involved in the regulation of sexual and agonistic behavior in this and other vertebrates. In one study, we examined the effect of sexual experience in females from an all-female incubation temperature (26 °C) and the male-biased incubation temperature (32.5 °C); all animals were two years at the time but one group was allowed to breed at one year of age whereas the other group was left sexually inexperienced (Crews et al., 1997). Fig. 5 illustrates that metabolic activity relative to overall brain activity differs in the same nuclei according to condition and that the functional landscape changes significantly according to incubation temperature of the embryo, but not according to the sexual experience of the individual in adulthood. That is, if one examines the effect of incubation temperature (comparison within columns), both inexperienced and experienced females from the male-biased incubation temperature show greater activity in the AH, NS, and SEP (but not in the POA, VMH, or PP, which are unchanged). These particular nuclei are

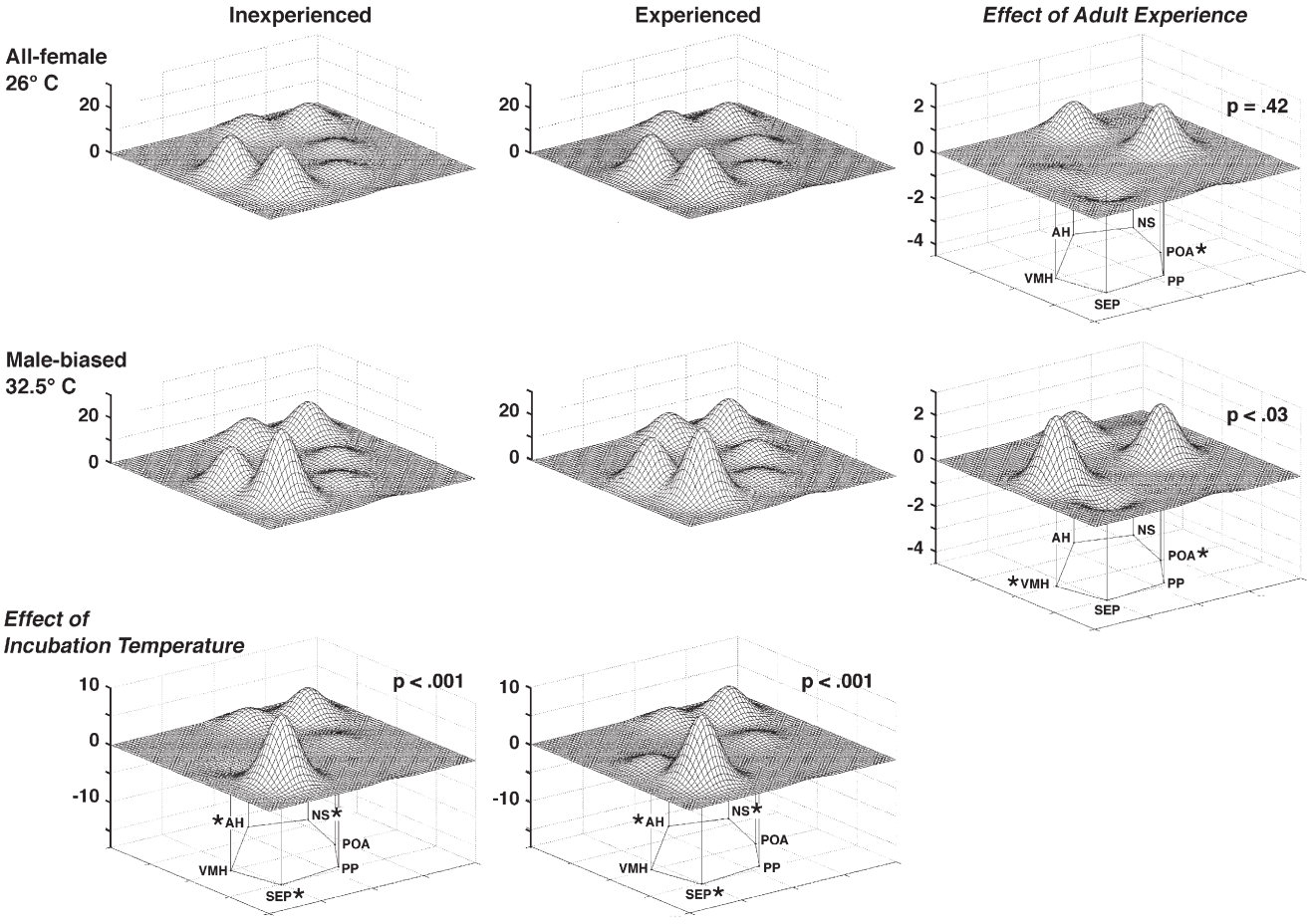

Fig. 5 – Incubation temperature modifies the abundance of cytochrome oxidase in limbic nuclei subserving sociosexual behavior in the adult female leopard gecko (*E. macularius*). Illustrated are means of cytochrome oxidase abundance relative to background in each nucleus of each of four groups. Eggs were incubated at one of two temperatures (all-female or 26 °C and male-biased or 32.5 °C) but the hatchlings were raised at identical temperatures. At one year of age females were allowed to bread for one reproductive season (experienced) or remained inexperienced (inexperienced). Note that in both sexually inexperienced and experienced groups (columns) comparison of the two different incubation temperatures results in a significant percentage increase in most, but not all, nuclei relative to overall brain activity. This in turn results in significant differences in the overall circuit. Within each incubation temperature (rows), however, adult sexual experience modifies the effect of embryonic incubation temperature in only the POA (all-female) or the POA and VMH (male-biased) nuclei. The effect of experience on the overall circuit is not significant at the all-female temperature, and only marginally significant at the male-biased temperature. Values are average cytochrome oxidase abundance in identified cell nuclei relative to background. Data derived from Crews et al. (1997). Brain nuclei: ventromedial hypothalamus (VMH); anterior hypothalamus (AH); nucleus sphericus (NS); preoptic area (POA); periventricular preoptic area (PP); and septum (SEP). Bottom row reveals effect of embryonic temperature; peaks above the plane indicate values are greater at the male-biased incubation temperature. Right column reveals effect of adult experience; peaks above plane indicate values are greater in sexually experienced individuals. Asterisk indicates significant differences in particular nuclei.

centrally involved in maturation of the hypothalamus–pituitary–gonadal (AH) and the hypothalamus–pituitary–adrenal (NS and SEP) axes. A different picture emerges when comparing inexperienced and experienced females from within each incubation temperature (comparison within rows). In this instance, adult sexual experience modifies the effect of embryonic incubation temperature in the POA in the all-female incubation temperature and in the POA and VMH in the male-biased incubation temperature, but to a lesser degree (note difference in scale). These particular nuclei are major integrative areas for hormonal effects on sexual behavior. It is of particular interest that the neural network is not significantly affected in females from the all-female incubation temperature, and only marginally so at the male-biased incubation temperature. Finally, it is worth noting that not all brain nuclei are affected by these embryonic or adult treatments (e.g., PP). What this means is that in the leopard gecko incubation temperature has a more profound effect on brain organization than does adult sexual experience. A different situation is observed in mammals, as we will see below.

5.2. *Sex ratio of the litter and its consequences on maternal behavior and, in turn, the sexual behavior of the offspring when adult in the rat*

Our work with rats has focused on the sex ratio of the litter and how this might modify maternal care and, in turn, the adult sociosexual behavior exhibited by the offspring from these litters. Male sexual behavior has been associated with the amount of maternal licking received early in life (reviewed in Moore, 1995). Males who receive less anogenital stimulation from their mothers when young have longer inter-intromission intervals, longer ejaculation latencies and post-ejaculatory intervals, and require more intromissions before their first ejaculation on their first sexual experience (Moore, 1984). But natural litters vary dramatically in their sex ratio, raising the question whether sex ratio supersedes this effect.

To evaluate the importance of sex ratio, litters were reconstituted shortly after birth to be either female-biased (2 males: 6 females) or male-biased (6 males: 2 females) while controlling for the sex ratio in the intrauterine environment. As adults, one male from each litter type was given sexual experience consisting of ten 30-min encounters with a hormonally primed stimulus female, whereas one male sibling remained sexually inexperienced. We found that males in both types of biased litters received more licking than did their female siblings and this did not vary as a function of the sex ratio (de Medeiros et al., 2005). However, the sex ratio of the litter during the postnatal period did have an effect on the sexual behavior of male offspring when they grew up. Males who grew up with more female than male siblings (female-biased litter) exhibited fewer mounting behaviors compared to males who were reared with more males (male-biased litter), but no differences in intromissions or ejaculation latency, suggesting that these males may, in fact, have been more efficient copulators. Interestingly, the mounting difference may not relate directly to the males' behavior, or attraction towards the female. Rather, it may instead reflect a change in the male's attractiveness because these males were also solicited less by stimulus females than were males from male-biased litters.

Not only is the behavior of adult males modified by the sex ratio of their litter, the abundance of COX in various limbic nuclei that regulate this behavior was affected differentially (Matthews et al., 2005) (Fig. 6). However, because not all the brains of animals tested for behavior have yet been processed, these COX results must be considered preliminary. Sexual experience results in an increase in COX in the mPOA in males reared in both female-biased and male-biased litters and it was the only neural site to show such consistent experience-induced differences. This is, of course, of interest because the mPOA is central to the expression of sexual behavior in male rats and the site that is most likely to undergo change with a sexual experience. In terms of the effects of sex ratio of the litter in the pattern of COX across neural sites, preliminary results suggest that sexually experienced males raised in female-biased litters, as opposed to those reared in male-biased litters, tend to show higher COX levels in most of the brain nuclei, although the extent of difference varies across site. This female-bias enhancement of metabolic activity will be analyzed along with the behavioral outcomes, to determine whether the brain changes could explain the findings that males raised in female-biased litters, when compared to males raised in male-biased litters, may require fewer mounts to achieve ejaculation.

5.3. *Litter sex ratio and genotype ratio influences adult behavior in knockout mice*

Behavioral phenotypes of knockout or transgenic mice are often interpreted as the effects of the absence or over-expression of a gene product. It is typical that researchers commonly ignore the sex and genotype ratios of the litters in which the animals tested were reared. For example, the estrogen receptor α knockout (ERKO) mouse has been the subject of extensive behavioral research, with the common finding that KO females are very aggressive and will mount intruder female mice whereas KO males are not aggressive toward male opponent mice and show reduced levels of male sexual behavior. However, there is significant variability in the responses of individuals, raising the question whether the composition of the litter in which the individual is raised might contribute to its later behavior.

We have examined this question of the contribution of litter sex ratio and genotype ratio by mating mice heterozygous (HTZ) for a null mutation of ERα, and then sexing and genotyping the offspring within two days of birth. Litters are then reconstituted to form same-sex litters of equal numbers of KO and wild-type (WT) individuals such as (1) same sex, mixed genotype female litters (all females, but half having a WT genotype and the other half having a KO genotype - FW/FK and FK/FW); or (2) same sex, mixed genotype male litters (all males, but half having a WT genotype and the other half having a KO genotype - MW/MK and MK/MW). The individuals were then tested as adults in a standard resident–intruder paradigm. In this manner the effect of genotype is evident without the potential confound of the presence of

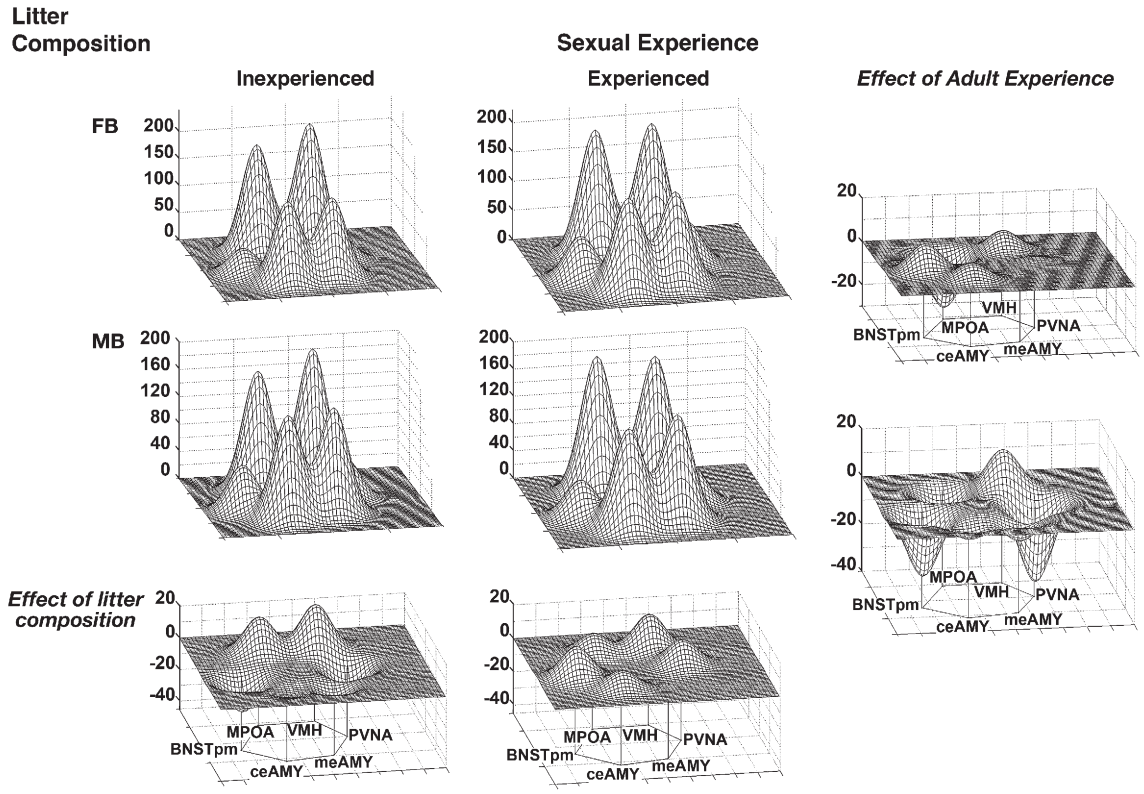

Fig. 6 – Litter composition influences how the limbic landscape changes after experience in adult male rats. Litters were constituted at birth to be either female-biased (FB, 2 males: 6 females) or male-biased (MB, 6 males: 2 females). As adults, one male from each litter type was given sexual experience consisting of ten 30-min encounters with a hormonally primed stimulus female, whereas one male sibling remained sexually inexperienced. Values are average cytochrome oxidase abundance in identified cell nuclei relative to background. Note that in both inexperienced and experienced groups (columns) comparison of the two litter compositions there is a significant increase in most, but not all, nuclei relative to overall brain activity and, further, that the functional landscape of the overall circuit varies depending upon adult experience. The effect of experience within each litter composition (rows) similarly results in dramatically different functional landscapes. Brain nuclei abbreviations: posterior medial bed nucleus of the stria terminalis (BNSTpm); medial preoptic area (MPOA); ventromedial hypothalamus (VMH); anterior paraventricular nucleus (PVNa); medial amygdala (meAMY); and central amygdala (ceAMY). Bottom row reveals effect of litter composition and valleys below the plane indicate values are greater in the MB litters. Right column reveals effect of adult experience and peaks above plane indicate values are greater in sexually experienced individuals whereas valleys indicate values greater in sexually inexperienced individuals.

the opposite sex in the litter. The consequence is that the behavioral differences between the genotypes are more sharply defined than reported previously, with KO females displaying only aggressive behavior, whereas their WT littermates display only mounting behavior; both aggression and mounting behavior are greatly reduced in ERKO males (Crews et al., 2004). These data suggested that litter composition influenced the development of sociosexual behaviors in ERKO mice.

We recently extended this study and tested WT females raised in three different litter compositions: FW/FW (all WT females), FW/FK (WT females raised with KO females), and FW/MW (WT females raised with WT males), as well as other litter compositions that will not be detailed here. WT females raised in these litters were then tested as before in the standard resident–intruder test. WT females from both the FW/FK and the FW/MW show lower contact times compared to females reared in FW/FW litters (Ogawa et al., 2005). It was interesting to find that although WT females from the litters containing KO females and the WT male littermates exhibit similar deficits in behavior, the neural network subserving sociosexual behavior vary in different ways (Fig. 7). For example, the presence of male sibs in the litter results to significant differences from all female WT litters (FW/FW) in the corticomedial amygdala coAMY), central medial preoptic area (C MPO), the paraventricular nucleus (PVN), and the main bed nucleus of the stria terminalis (BNSTma), but only the C MPO was different in WT females raised with other WT females versus those raised with KO female sibs. This difference is seen particularly clearly when we look at the difference in the limbic landscapes according to the type of sib. Taken together, these findings indicate that in knockout mice, the litter composition during the preweaning period affects the development of behavior and the neural network

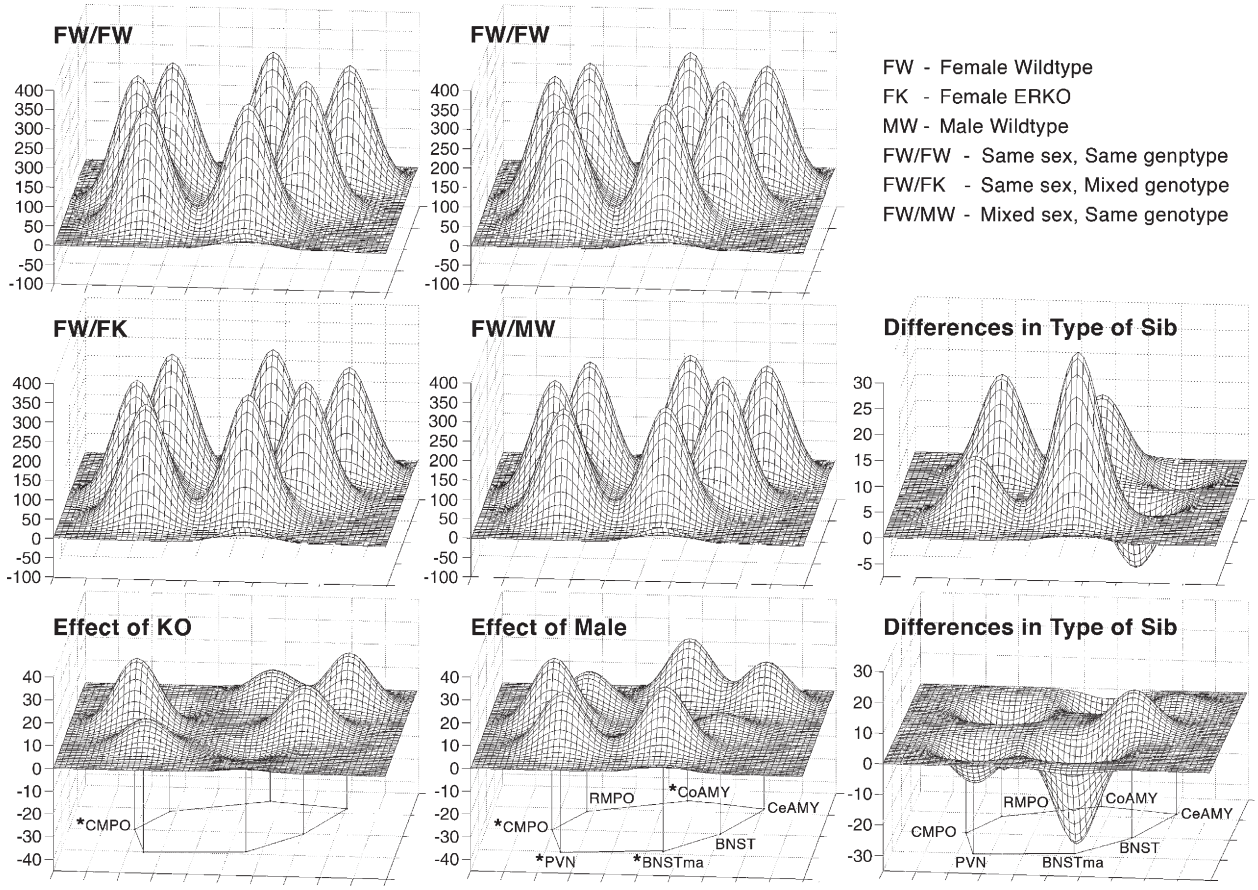

Fig. 7 – Both sex ratio and genotype ratio influences how the limbic landscape changes after experience in adulthood in female mice. Litters were (1) same sex, same genotype litters (all individuals being female and having a WT genotype—FW/FW); (2) same sex, mixed genotype litters (all females, but with half having a WT genotype and the other half having a knockout genotype—FW/FK); or (3) mixed sex, same genotype litters (all individuals having a WT genotype, but half being female and the other half being male—FW/MW). All mice were produced by mating of females heterozygous for the estrogen receptor α gene, producing the knockout (ERKO) and male and female wild-type mice (MW and FW, respectively). Values are average cytochrome oxidase abundance in identified cell nuclei relative to background. For sake of comparison landscape maps for FW/FW litter are double plotted (top row). Note the Effect of KO and the Effect of male (bottom left and middle column) functional landscapes. This difference is highlighted in the right column, the Differences in Types of Sib, plotted in mirror images to illustrate differences in specific nuclei. Brain nuclei abbreviations: central medial preoptic area (C MPO); rostral medial preoptic area (RMPO); corticomedial amygdala (coAMY); central amygdala (ceAMY); bed nucleus of the stria terminalis (BNST); main bed nucleus of the stria terminalis (BNSTma); and paraventricular nucleus (PVN). Asterisks indicate significant differences in specific nuclei.

responsible for the regulation of emotional behaviors in individuals later in adulthood.

6. Conclusions

In vertebrates, a handful of genes is known to be centrally involved in the processes of sex determination and gonadal differentiation. About the same number of brain nuclei comprise the neural network that underlies vertebrate social behaviors. In both processes a discrete number of genes and brain nuclei, respectively, are not only involved in the final product, but they all have established interactions. This makes it feasible to depict the dynamic interaction among components of each system as well as how that system might change through time or manipulation.

Several instances from our work are used to present a method for depicting and quantifying such changes in gene expression in the gonad and metabolic activity in limbic nuclei. In particular, we show how in the turtle the environment shapes a network of genes that play the central role in temperature-dependent sex determination and gonadal differentiation. As in other vertebrates, these early gonadal events organize the brain, in this instance according to incubation temperature more so than gonadal sex, but they are not fixed. Sexual experience later in adulthood modifies further the neural network that underlies sociosexual behavior.

How the early hormonal environment organizes the sexual differentiation process of the neuroendocrine substrates of reproductive behaviors has dominated the field of behavioral neuroscience. Although such work has yielded important insights into the regulation of sociosexual behaviors, it is time to move toward understanding how postnatal and adult experiences interact to further shape brain–behavior relationships within individuals of the same sex. Many studies with mammals have documented the importance of maternal care in influencing adult behavior and how these effects can span generations. But how maternal care might be influenced by the sex ratio of the litter and how this, in turn, affects how individual pups are treated have not been considered. We demonstrate that this experience determines how individuals will respond later in adulthood to sexual experience. Further, the sex ratio of the litter interacts with the ratio of the different genotypes in knockout mice to influence the emergence of different behavioral phenotypes and their underlying neural correlates.

Taken together, these data attest to the validity of the proposition "Genetics proposes, epigenetics disposes" (Medawar and Medawar, 1983; see also Crews and McLachlan, 2006).

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REFERENCES

Akaike, H., 1974. A new look at the statistical model identification. IEEE Transaction Automat. Contr. AC 19, 716–723.

Behringer, R.R., Cate, R.L., Froelick, G.J., Palmiter, R.D., Brinster, R.L., 1990. Abnormal sexual development in transgenic mice chronically expressing Müllerian inhibiting substance. Nature 345, 167–170.

Behringer, R.R., Finegold, M., Cate, R.L., 1994. Müllerian-inhibiting substance function during mammalian sexual development. Cell 79, 415–425.

Crews, D., 1993. The organizational concept and vertebrates without sex chromosomes. Brain, Behav. Evol. 42, 202–214.

Crews, D., 1996. Temperature-dependent sex determination, the interplay of steroid hormones and temperature. Zool. Sci. 13, 1–13.

Crews, D., McLachlan, J.A., 2006. Epigenetics, evolution, endocrine disruptors, health and disease. Endocrinology 147, S4–S10 (Supplement).

Crews, D., Bergeron, J.M., Bull, J.J., Flores, D., Tousignant, A., Skipper, J.K., Wibbels, T., 1994. Temperature-dependent sex determination in reptiles, proximate mechanisms, ultimate outcomes, and practical applications. Dev. Genet. 15, 297–312.

Crews, D., Coomber, P., Gonzalez-Lima, F., 1997. Effects of age and sociosexual experience on the morphology and metabolic capacity of brain nuclei in the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination. Brain Res 758, 169–179.

Crews, D., Sakata, J., Rhen, T., 1998. Developmental effects on intersexual and intrasexual variation in growth and reproduction in a lizard with temperature-dependent sex determination. Comp. Biochem. Physiol., Part C 119, 229–241.

Crews, D., Fleming, A., Willingham, E., Baldwin, R., Skipper, J.K., 2001. Role of steroidogenic factor 1 and aromatase in temperature-dependent sex determination in the red-eared slider turtle. J. Exp. Zool. 290, 597–606.

Crews, D., Fuller, T., Mirasol, E.G., Pfaff, D.W., Ogawa, S., 2004. Postnatal environment affects behavior of adult transgenic mice. Exp. Biol. Med. 229, 935–939.

de Medeiros, C.B., Rees, S.L., Rees, S., Llinas, M., Bardo, B., Crews, D., Fleming, A.S., 2005. Effects of postnatal litter sex composition on adult sexual behavior in male rats. Abstr.-Soc. Neurosci. 31, 321.11.

Fitzmaurice, G.M., Laird, N.M., Ware, J.H., 2004. Applied Longitudinal Analysis. John Wiley and Sons, New York.

Fleming, A., Crews, D., 2001. Estradiol and incubation temperature modulate regulation of steroidogenic factor 1 in the developing gonad of the red-eared slider turtle. Endocrinology 142, 1403–1411.

Fleming, A., Wibbels, T., Skipper, J.K., Crews, D., 1999. Developmental expression of steroidogenic factor 1 in a turtle with temperature-dependent sex determination. Gen. Comp. Endocrinol. 116, 336–346.

Fuller, T., Sarkar, S., Crews, D., 2005. The use of norms of reaction to analyze strain differences in mice and rats in behavioral neuroscience research. Neurosci. Biobehav. Rev. 29, 445–456.

Gottesman, I.I., 1997. Twins: en route to QTLs for cognition. Science 276, 1522–1523.

Grossman, A.W., Churchill, J.D., McKinney, B.C., Kodish, I.M., Otte, S.L., Greenough, W.T., 2003. Experience effects on brain development: possible contributions to psychopathology. J. Child Psychother. 44, 33–63.

Keselman, H.J., Algina, J., Kowalchuk, R.K., Wolfinger, R.D., 1999. A comparison of recent approaches to the analysis of repeated measurements. Br. J. Math. Stat. Psychol. 52, 63–78.

Koopman, P., 2001. The genetics and biology of vertebrate sex determination. Cell 105, 843–847.

Lehrman, D.S., 1953. A critique of Konrad Lorenz's theory of instinctive behavior. Q. Rev. Biol. 28, 337–373.

Liang, K.Y., Zeger, S.L., 1986. Longitudinal data analysis using generalized linear models. Biometrika 73, 13–22.

Lindstrom, M.J., Bates, D.M., 1988. Newton–Raphson and EM algorithms for linear mixed effects models for repeated measures data. J. Am. Stat. Assoc. 83, 1014–1022.

Loffler, K.A., Zarkower, D., Koopman, P., 2003. Etiology of ovarian failure in blepharophimosis ptosis epicanthus inversus syndrome, FOXL2 is a conserved, early-acting gene in vertebrate ovarian development. Endocrinology 144, 3237–3243.

Luo, X., Ikeda, Y., Parker, K., 1994. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77, 481–490.

Matthews, R.N., Gonzalez-Lima, F., Crews, D., Rees, S., de Medeiros, C.B., Fleming, A.S., 2005. Litter composition alters phenotypic and neural plasticity in rat. Abstr.-Soc. Neurosci. 31, 892.13.

Medawar, P., Medawar, J., 1983. Aristotle to Zoos. Harvard Univ. Press, Cambridge.

Moore, C.L., 1995. Maternal contributions to mammalian reproductive development and divergence of males and females. In: Slater, P.J.P., Rosenblatt, J.S., Snowdon, C.T., Milinski, M. (Eds.), Advances in the Study of Behavior. Academic Press, New York, pp. 47–118.

Newman, S.W., 1999. The medial extended amygdala in male reproductive behavior, A node in the mammalian social behavior network. Ann. N. Y. Acad. Sci. 877, 242–257.

Nijhout, H.F., 2003. The importance of context in genetics. Am. Sci. 91, 416–418.

Ogawa, S., Luk, S.L., Murphy, L., Matthews, N., Pfaff, D.W., Tomihara, K., Soga, T., Crews, D., 2005. Effects of the litter composition during pre-weaning period on the development of anxiety-related behaviors in mice. Abstr.-Soc. Neurosci. 31, 892.14.

Oreal, E., Pieau, C., Mattei, M.-G., Josso, N., Picard, J.-Y., Carre-Eusebe, D., Magre, S., 1998. Early expression of AMH in chicken embryonic gonads precedes testicular SOX9 expression. Dev. Dyn. 212, 522–532.

Oreal, E., Mazaud, S., Picard, J.-Y., Magre, S., Carre-Eusebe, D., 2002. Different patterns of anti-Müllerian hormone expression, as related to DMRT1, SF-1, WT1, GATA-4, Wnt-4, and Lhx9 expression, in the chick differentiating gonads. Dev. Dyn. 225, 221–232.

Putz, O., Crews, D., 2006. Embryonic origin of mate choice in a lizard with temperature-dependent sex determination. Dev. Psychobiol. 48, 29–38.

Raymond, C.S., Shamu, C.E., Shen, M., Seifert, K., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature 391, 691–695.

Raymond, C.S., Kettlewell, J.R., Hirsch, B., Bardwell, V.J., Zarkower, D., 1999. Expression of Dmrt1 in the genital ridge of mouse and chicken embryos suggests a role in vertebrate sexual development. Dev. Biol. 215, 208–220.

Rhen, T., Sakata, J.T., Crews, D., 2005. Gonadal sex and incubation temperature modulate sex steroid hormone levels and secondary sex structures during leopard gecko development. Gen. Comp. Endocrinol. 142, 289–296.

Sakata, J.T., Crews, D., 2004. Developmental sculpting of phenotype and plasticity. Neurosci. Biobehav. Rev. 28, 95–112.

Sakata, J.T., Crews, D., Gonzalez-Lima, F., 2005. Behavioral correlates of differences in neural metabolic activity. Brain Res. Rev. 48, 1–15.

Schwarz, G., 1978. Estimating the dimension of a model. Ann. Stat. 6, 461–464.

Smith, C., McClive, P.J., Western, P.S., Reed, K., Sinclair, A.H., 1999a. Conservation of a sex determining gene. Nature 402, 601–602.

Smith, C., Smith, M., Sinclair, A.H., 1999b. Gene expression during gonadogenesis in the chicken embryo. Gene 234, 395–402.

Torres-Maldonando, L., Piedra, A.L., Moreno-Mendoza, N., Valencia, A.M., Martinez, A.M., Merchant-Larios, H., 2002. Expression profiles of Dax1, Dmrt1, and Sox9 during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*. Gen. Comp. Endocrinol. 129, 20–26.

Waddington, C.H., 1957. The Strategy of the Genes. Middle, West Drayton.

Western, P.S., Harry, J.L., Graves, J.A.M., Sinclair, A.H., 1999. Temperature-dependent sex determination in the American alligator, AMH precedes Sox9 expression. Dev. Dyn. 216, 411–419.

Western, P.S., Harry, J.L., Marshall Graves, J.A., Sinclair, A.H., 2000. Temperature-dependent sex determination in the American alligator, expression of SF1, WT1, and DAX1 during gonadogenesis. Gene 241, 223–232.

Young, W.C., 1961. The hormones and mating behavior. In: Young, W.C. (Ed.), Sex and Internal Secretions, vol. II. Williams and Wilkins, Baltimore, pp. 1173–1239.