KIN RECOGNITION AND MHC DISCRIMINATION IN AFRICAN CLAWED FROG (*XENOPUS LAEVIS*) TADPOLES

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ABSTRACT

Kin-recognition abilities, first demonstrated 25 years ago in toad tadpoles, now appear to be widespread among amphibians. In some vertebrates kin recognition is based, at least in part, on highly polymorphic major histocompatibility complex (MHC) genes. Besides protecting animals from disease resistance, MHC genes regulate social behaviour. They allow relatives to recognise one another so that they can cooperate for mutual benefit. These two seemingly distinct functions of MHC genes may be integrally related, because animals need to outbreed to optimise the immune systems of their offspring. The ability to discriminate MHC-type is therefore likely to facilitate kin discrimination in tadpoles.

I tested association preferences of African clawed-frog (*Xenopus laevis*) tadpoles in a laboratory choice apparatus. As in other anuran species, I found that tadpoles at earlier developmental stages preferentially associate with unfamiliar siblings over unfamiliar non-siblings but that this preference reverses during development. Tadpoles approaching metamorphosis demonstrated a reversal in their preference; they preferentially school with non-kin rather than kin. The ontogenetic switch in larval schooling preferences coincides with the onset of thyroid hormone (TH) controlled development and may be indicative of decreased fitness benefits associated with schooling with kin at later developmental stages. These may result from an increase in intraspecific competition, predation, or disease susceptibilities of prometamorphic individuals. Alternatively, the kin avoidance behaviours observed at later larval stages might reflect disassociative behaviour that facilitates inbreeding avoidance at reproductive maturity. This is the first study to find a shift from an association preference for kin to non-kin during amphibian larval development.

Using allele-specific PCR techniques to MHC-type tadpoles, I tested association preferences among siblings based on shared MHC haplotypes. By using only full siblings in experimental tests, I controlled for genetic variation elsewhere in the genome that might influence schooling preferences. I found that *X. laevis* tadpoles discriminate among familiar

full siblings based on differences at MHC genes. Subjects from four families preferentially schooled with MHC-identical siblings over those with which they shared no or one haplotype. Furthermore, the strength of tadpoles' MHC-assortative schooling preferences significantly correlated with amino acid differences in the peptide-binding region (PBR) of both the MHC class I and II loci. Since MHC-PBR polymorphisms determine the pool of peptides that can serve as ligands for MHC molecules, these findings support the hypothesis that MHC peptide ligands mediate MHC type discrimination. As test subjects were equally familiar with all stimulus groups, tadpole discrimination appears to involve a self-referent genetic recognition mechanism whereby individuals compare their own MHC type with those of conspecifics.

I also found that non-MHC-linked genetic differences contribute to tadpole association preferences in tests that contrast MHC and kinship. Tadpoles did not discriminate between MHC-similar non-siblings and MHC-dissimilar siblings and preferentially associated with MHC-dissimilar non-siblings rather than MHC-similar non-siblings. Although the MHC may be not solely responsible for the genetically determined cues that direct tadpole association preferences, it certainly is important in facilitating discrimination among conspecifics in *X. laevis* tadpoles. MHC-based discrimination may be retained through ontogeny and serve to maintain MHC-polymorphisms by facilitating disassortative mating.

CONTENTS

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Chapter 1 : General Introduction

Many animals have been shown to recognise their kin. The ability to discriminate kin from non-kin even in the absence of prior social familiarity has been demonstrated in many anuran species in various contexts – most extensively in tadpole schooling (Waldman 2005). In fact, frogs were the first vertebrates shown to have kin recognition abilities (Waldman & Adler 1979). More recently, the major histocompatibility complex (MHC) has been shown to play a key role in kin recognition in fishes, lizards, birds, rodents and humans (Penn & Potts 1999; Bernatchez & Landry 2003; Piertney & Oliver 2006). In this thesis, I investigate kin and MHC-biased association preferences in *Xenopus laevis* tadpoles. MHC-biased behaviours never have been demonstrated in anurans, which have been otherwise model organisms for the study of kin recognition.

MHC-type discrimination may facilitate kin-discrimination, which in turn can facilitate nepotistic interactions among tadpoles. Theoretically, MHC loci are ideal candidates for a genetic basis of kin recognition, as they are highly polymorphic and generate cellular markers that facilitate self/non-self recognition in the immune system. Self/non-self recognition for the purposes of kin discrimination is analogous to the MHC's function in facilitating self/non-self immune recognition. Since the MHC serves the function of cellular recognition in vertebrates, it is feasible that it could also be involved in a similar kind of recognition on an organismic level, i.e. kin recognition. As MHC facilitated recognition leads to an adaptive immune response against pathogens, pathogen infected cells, or cells bearing dissimilar MHC molecules to ensure the survival of the organism (Klein & O'Huigin 1994), kin recognition may lead to socially cooperative behaviour among related individuals that results in increased inclusive fitness (Bernatchez & Landry 2003; Piertney & Oliver 2006). MHC loci are the only known loci with the necessary degree of polymorphism to function as effective self-markers that can facilitate both immune and individual discrimination in vertebrates, making the MHC the most likely loci to be involved in genetic kin recognition (Penn & Potts 1999; Bernatchez & Landry 2003; Piertney & Oliver 2006).

MHC-biased behaviours, and in particular MHC-disassortative mating, may have contributed to the maintenance of polymorphisms observed in most vertebrate populations (Penn & Potts 1999; Bernatchez & Landry 2003; Piertney & Oliver 2006). The MHC genetic diversity is in itself paradoxical because, given the fitness that the MHC confers, its allelic variation should be depleted by strong directional selection. However, additional pleiotropic effects, such as directing inbreeding avoidance or determining different disease susceptibilities, may help maintain MHC polymorphisms (Penn & Potts 1999; Hedrick 2002; Bernatchez & Landry 2003). Although MHC-disassortative mating is the only behavioural context that has direct implications to the evolution of the MHC itself, the underlying mechanism of MHC-linked kin recognition is likely to be relevant to other social contexts (Penn & Potts 1999; Bernatchez & Landry 2003; Milinski *et al.* 2005) – such as, in the case of this study, tadpole schooling. In the present thesis, I present original research in which I explored kin- and MHC-discrimination in *X. laevis* tadpoles.

MHC-biased behaviours have not yet been investigated in *Xenopus laevis*, or any other amphibians for that matter. Amphibians in general are excellent model organisms for the study of socially biased behaviours due to their complex life history as well as the fecundity of many amphibian species. Amphibians can be studied as larvae or as adults, in aquatic or terrestrial habitats, in various contexts that give us insight into the development of their behaviours that may be correlated with changes in ecological niche, anatomy, physiology or gene expression. *Xenopus* frogs can produce thousands of tadpoles in a single mating. Therefore an experimental assay based on tadpole kin recognition provides an efficient means to study MHC-based kin recognition.

Xenopus laevis is particularly well suited to studying MHC-based kin recognition as its olfactory organs have been described extensively (Reiss & Burd 1997; Hansen *et al.* 1998; Petti *et al.* 1999; Franco *et al.* 2001; Hagino-Yamagishi *et al.* 2004; Manzini & Schild 2004; Pinelli *et al.* 2004) and it only has four closely linked MHC loci with exceptionally high amino acid polymorphisms (i.e. a large number of amino acids differ between alleles)

(Flajnik *et al.* 1999a; Liu *et al.* 2002; Bos & Waldman 2006). The unique simplicity of the *X. laevis* MHC allows for the entire MHC region to be typed based on a single genetic marker. The ability to breed frogs in the lab to produce MHC-type variation within larval sibships provides the opportunity to control for other cues that may encode for kinship by investigating MHC-linked association preferences among siblings and among non-siblings.

In over 1400 120-min two-way choice tests in 11 different test conditions (with 2-4 sibship replicates), I investigated whether *X. laevis* tadpoles preferentially associate with kin and/or MHC-similar conspecifics. These studies lend insight into the potential evolutionary advantages and mechanisms involved in MHC-type and kin recognition. The body of this thesis consists of two review chapters (2 & 3), one methods chapter (4), and four data chapters (5-8). In the methods chapter I present the MHC-typing and choice test methods employed in the following four data chapters. The data chapters are written for submission to separate journals but make reference to the methods chapter for technical detail.

In Chapter 2 : The ecology and mechanisms of kin recognition, I discuss potential mechanisms by which organisms discriminate kin from non-kin as well as how kin recognition abilities may increase an individual's fitness.

In Chapter 3 : Adaptive immunity and MHC-biased behaviours, I discuss MHC mediated adaptive immunity, selection processes by which the extraordinary polymorphisms of the MHC are maintained, MHC-biased behaviours, how MHC-biased behaviours may have been selected for, and mechanisms by which MHC-type can be discriminated on an individual level. I also introduce the *Xenopus* MHC and discuss how the MHC may be involved in biasing social behaviour in *X. laevis* tadpoles.

In Chapter 4 : Materials and methods, I describe experimental methods used in the data chapters (5-8).

In Chapter 5 : Kin-recognition in African clawed frog (*Xenopus laevis*) tadpoles: Ontogeny and discrimination cues, I present and discuss experimental results of *X. laevis* kin/non-kin association preference tests:

(a) At different stages of larval development.

- Do *X. laevis* tadpoles discriminate unfamiliar siblings from unfamiliar non-siblings?
- Do *X. laevis* tadpoles from early and late developmental stages demonstrate similar association preferences between unfamiliar siblings and unfamiliar non-siblings?

(b) In which either only visual or only waterborne cues were available to subjects.

- Do *X. laevis* tadpoles discriminate unfamiliar siblings from unfamiliar non-siblings in the absence of visual cues?
- Do *X. laevis* tadpoles discriminate unfamiliar siblings from unfamiliar non-siblings in the absence of waterborne cues?

In Chapter 6 : Self-referent MHC-linked genotype matching in *Xenopus laevis* tadpoles, I present and discuss experimental results of *X. laevis* association preference tests among siblings based on shared MHC-type.

- Do *X. laevis* tadpoles preferentially associate with MHC-identical siblings over MHC-different siblings?

In Chapter 7 : MHC-type discrimination in *Xenopus laevis* tadpoles correlates with the number of amino acid differences in the peptide binding region of the MHC class I and II, I present and discuss experimental results of *X. laevis* association preference tests among siblings based on

(a) Variable numbers of shared MHC haplotypes.

- Do *X. laevis* tadpoles discriminate among siblings based on single-haplotype differences at the MHC?

(b) Amino acid similarities in the peptide-binding region (PBR) of both MHC class I and MHC class II loci.

- Do *X. laevis* tadpole MHC-assortative association preferences correlate with amino acid differences in the PBR of the MHC class I and II loci?

In Chapter 8 : Evidence for *X. laevis* tadpole association preferences associated with genetically determined cues that are unlinked to the MHC, I present and discuss experimental results of *X. laevis* association preference tests

(a) Between MHC-identical non-siblings and siblings with which test subjects share no MHC haplotypes.

- Do *X. laevis* tadpoles preferentially associate with either MHC-identical non-siblings or MHC-different siblings?

(b) Among non-siblings that are either MHC-identical or share no MHC haplotypes with test subjects.

- Do *X. laevis* tadpoles preferentially associate with MHC-identical non-siblings over MHC-different non-siblings?

In Chapter 9 : General Discussion, I review and discuss the main findings of this thesis.

Chapter 2 : The Ecology and Mechanisms of Kin Recognition

Kin recognition is the ability to assess genetic relatedness. Kin discrimination is the differential treatment of conspecifics based on cues that correlate with genetic relatedness. Kin-biased behaviour refers to the types of behaviours in which kin discrimination occurs, which are context dependent. Many animals have been shown to recognise their kin. The ability to discriminate kin from non-kin even in the absence of prior social familiarity has been demonstrated in many anuran species in various contexts – most extensively in tadpole schooling (Waldman 2005). In fact, frog tadpoles were among the first vertebrates shown to have kin recognition abilities (Waldman & Adler 1979).

The ability to distinguish kin from non-kin may facilitate nepotistic interactions. Depending on the social and ecological contexts, kin selection theory predicts that directing intraspecific interactions towards or away from kin may increase an individual's inclusive fitness and will be selected for, even if the behaviour decreases an individual's direct fitness (Hamilton 1964). When the benefits outweigh the costs of associating with kin, an individual may optimise its inclusive fitness and/or direct fitness by associating with kin (Hamilton 1964). Conversely, when the costs outweigh the benefits of associating with kin, an individual may optimise its fitness by avoiding kin association (Hamilton & May 1977).

Kinship and Group Living

Species that aggregate, such as many species of anuran larvae, are good model organisms to examine kin-biased social interactions. Accordingly, there is a large body of laboratory research that investigated kin association preferences of anuran tadpoles (Waldman 2005; Gramapurohit *et al.* 2006). Consistent with kin selection theory, most of the published studies found that tadpoles can discriminate kin from non-kin and preferentially associate with kin (Waldman 2005; Gramapurohit *et al.* 2006).

The kin-biased behaviours observed in most laboratory experiments may however differ in the wild, depending on the ecological context. Using dye-marked American toad (*Bufo americanus*) tadpoles to indicate sibship identity, Waldman (1982) found significant differences in sibship composition in natural outdoor ponds. Consistent with kin-association preferences observed in laboratory studies on wood frog (*Rana sylvatica*) tadpoles (Waldman 1984; Cornell *et al.* 1989; Fishwild *et al.* 1990; Gamboa *et al.* 1991a; Gamboa *et al.* 1991b; Rautio *et al.* 1991), Halverson *et al.* (2006) found that *R. sylvatica* tadpoles clumped with their siblings or half-siblings in one pond. However, in another pond they found the opposite spatial distribution of kin groups in which tadpoles were nonrandomly dispersed from their kin; kin were hyperdispersed (Halverson et al., 2006). Though somewhat inconclusive, these results highlight the possibility that kin-associative behaviour may depend on localised contexts that may change the balance between the associated fitness benefits and costs.

The propensity to school, even with unrelated conspecifics, may be selectively advantageous in certain contexts. Individuals in groups may compete more effectively for resources (e.g. food), may be able to regulate their environments for their mutual benefit (e.g. thermoregulation), and may detect, avoid or deter predators more effectively (Hamilton 1971; Alexander 1974; Waldman 1982; Blaustein & Waldman 1992; Hokit & Blaustein 1997). Individuals that preferentially associate with kin might accrue additional benefits, especially when the costs of group living are inequitably distributed among members in a group (Waldman 1982, 1988; Blaustein & Waldman 1992). Individuals located at the edge of a school may be more vulnerable to predation than those in the centre that they shield (Waldman 1982). Tadpoles at the edge of a school would therefore increase their inclusive fitness by preferentially schooling with close kin (Black 1970; Katz *et al.* 1981). Tadpoles injured by a predator may warn surrounding individuals of predator presence. The production and release of warning cues, such as alarm pheromones found in *Bufo* tadpoles (Hrbacek 1950; Kulzer 1954; Pfeiffer 1966; Waldman 1986; Lefcort 1998) and many ostariophysan fishes (von Frisch 1941; Pfeiffer 1977), increases an individual's inclusive fitness only when kin benefit disproportionately (Waldman 1982). Similarly, the general

benefits of schooling in groups such as thermoregulation and foraging efficiency can increase an individual's inclusive fitness when they are disproportionately shared with kin (Hokit & Blaustein 1997).

Costs may be associated with social aggregation in certain contexts, including increased competition, cannibalisation, predation, susceptibility to disease, and inbreeding (Hamilton & May 1977; Shykoff & Schmid-Hempel 1991; Pfennig & Collins 1993; Garrett & Mundt 1999; Halverson *et al.* 2006). The propensity of some anuran larvae to school has been found to diminish with more even food distribution, the presence of predators, and lower temperature variability (Hokit & Blaustein 1997). In such contexts, kin avoidance may increase an individual's fitness by avoiding competition with kin (Hamilton & May 1977), by spreading the risk of predation by certain predators (Halverson *et al.* 2006), or reducing the spread of disease among more genetically diverse conspecifics (Shykoff & Schmid-Hempel 1991; Garrett & Mundt 1999).

Intraspecific Interactions: Kinship and Growth Rates

Competitive interactions within larval schools are predicted to differ among kin and non-kin. Indeed, the kinship composition of social groups can influence rates of growth and development (Jasieñski 1988; Waldman 1988; Smith 1990; Blaustein & Waldman 1992; Hokit & Blaustein 1997; Pakkasmaa & Aikio 2003; Pakkasmaa & Laurila 2004; Waldman 2005). Various laboratory-controlled studies indicate that tadpole growth is differentially regulated by exposure to siblings and non-siblings. Depending on the species of tadpoles and the ecological context of the experiment, the results of kin association can be drastically different. Tadpoles reared with kin can grow to be larger (Jasieñski 1988; Smith 1990; Pakkasmaa & Aikio 2003; Pakkasmaa & Laurila 2004), or to be smaller (Shvarts & Pyastolova 1970; Hokit & Blaustein 1994) than tadpoles reared with non-kin, or may show no difference in size (Travis 1980; Pakkasmaa & Laurila 2004). Tadpoles reared with kin can

either be more variable (Waldman 2005) or less variable (Travis 1980; Jasieñski 1988; Pakkasmaa & Aikio 2003) in size than those reared in mixed groups.

Greater growth variability within kin groups can be a function of increased nepotistic self-control (Waldman 1991) in species that form tight aggregates. Large, growing tadpoles can reduce the growth rate of smaller conspecifics by releasing growth inhibiting factors into the water (Richards 1958; Steinwascher 1979; Bardsley & Beebee 2000) or through behavioural interactions (Gromko *et al.* 1973; John & Fenster 1975; Waldman 1982; Smith 1990). Although inclusive fitness presumably would be maximized by directing growth inhibition effects toward non-kin rather than toward kin, selection may be acting on the specificity with which individuals respond to the regulatory effects (Waldman 1982). Indeed, the relatedness to individuals releasing the inhibitors can influence tadpole responses to the inhibitors. Substances released by large *Rana arvalis* tadpoles have a greater inhibitory effect on the growth of smaller siblings than on that of smaller non-siblings (Shvarts & Pyastolova 1970). Consistent with a kin selection model, factors released by small larvae may increase the growth rate of their larger kin (Shvarts & Pyastolova 1970; Steinwascher 1979)**.** Furthermore, at metamorphosis these larger tadpoles may release chemicals that accelerate the growth of less developed individuals (Waldman 1982). Waldman (1982) suggests that the responsiveness to growth regulating factors from kin might also be influenced by an individual's stage of development and likelihood of survival to metamorphosis. Nepotistic self-control of smaller tadpoles within kin groups may thus function to increase their inclusive fitness by increasing the direct fitness of larger kin that have a better chance of survival to metamorphosis.

Alternatively, reduced growth variability within kin groups can function to direct competition and intraspecific predation (cannibalism) away from kin (Hamilton & May 1977; Pfennig & Collins 1993; Hokit *et al.* 1996; Pakkasmaa & Aikio 2003) in species that form loose aggregates, such as *Xenopus laevis* (Wassersug & Hessler 1971; Wassersug *et al.* 1981). Reduced competition among siblings might reduce stress levels, thereby limit

immunosuppression, and thus help protect tadpoles from infectious disease. Barribeau (2007) found that *X. laevis* tadpoles display reduced variation in growth within kin groups. An additional benefit of reduced size variation among kin in *X. laevis* may be to reduce the chances of cannibalising kin after metamorphosis. Newly metamorphosed frogs frequently cannibalise nearby tadpoles (Parker *et al.* 1947; Tinsley *et al.* 1996; Measey 1998) but metamorphs are gape-limited; large tadpoles are often bigger than the new froglets. Recent metamorphs within kin groups that are closer in size to their tadpole siblings would be unable to cannibalise them.

Mechanisms of Kin Recognition

To discriminate kin from non-kin, individuals must be able to assess the genetic relatedness of conspecifics. There are a variety of cues that correlate with relatedness that may facilitate such kin recognition (Gamboa *et al.* 1991a; Mateo 2002)**.** Kin recognition may be indirect, when animals rely upon contextual features such as aspects of the environment that are predictably associated with kin, or direct, when based on the perception and evaluation of relatives' phenotypic traits (Waldman 1988). If kin are encountered in a variety of social contexts in which unrelated individuals are also likely to be encountered, kin discrimination is likely to be the result of a direct kin recognition mechanism (Waldman 1988). For direct kin recognition to occur, individuals must bear kinship labels that can be perceived by conspecifics as kin or non-kin based on a comparison to an internal template of kin traits. Kinship labels may be either environmental or genetic in origin and kin templates may be learned or genetically determined. The process by which an individual learns the phenotypes associated with kinship and stores a representation of these traits in memory as a kin template is referred to as phenotype matching (Waldman *et al.* 1988). Phenotype matching occurs when specific individuals previously encountered are recognized, or when phenotypic traits that correlate with kinship are learned in contexts that reliably predict relatedness (Waldman *et al.* 1988). The latter form of phenotype matching can result from

familiarity early in development with conspecifics within aggregations (i.e., nests, litters, and clutches) that are likely to represent closely related individuals (i.e., parents, siblings) (Waldman *et al.* 1988). Alternatively, since an individual's own phenotypic traits generally reflect its genotype more accurately than those of close kin (Mateo & Johnston 2000), phenotype matching may result from a self-referent mechanism by which an animal uses some aspect of its own phenotype as a referent to identify its relatives (Holmes & Sherman 1982; Mateo & Holmes 2004). Kin recognition mediated by self-referent phenotype matching does not depend on prior experience with related individuals (Waldman 1981), but could instead be based on learning one's own phenotype (Holmes & Sherman 1982) or result more directly from a 'genetic recognition mechanism' (Hamilton 1964; Dawkins 1976).

Self-Referent Phenotype Matching

Some studies suggest that kin recognition abilities in frog tadpoles are mediated by self-referent phenotype matching (Waldman 1981, 1986; Cornell *et al.* 1989; Hepper & Waldman 1992). American toad (*Bufo americanus*) tadpoles reared in mixed kin-group tanks spent more time orienting toward their unfamiliar siblings than toward familiar non-siblings (Waldman 1986). Such results suggest that traits of conspecifics encountered during an early sensitive period are incorporated into recognition templates that serve as models for phenotype matching (Waldman 1981, 1986). Further evidence for self-referent phenotype matching in anuran amphibians stems from studies in *B. americanus* (Waldman 1981) and *Rana cascadae* (Blaustein & O'Hara 1981) tadpoles in which naïve individuals, that were reared in isolation, discriminated siblings from non-siblings. *B. americanus* (Waldman 1981) and *Rana sylvatica* (Cornell *et al.* 1989) tadpoles reared in isolation discriminated paternal – but not maternal – half-siblings from full-siblings. Maternal biases in kin recognition abilities are also evident in *Xenopus laevis* tadpoles (Locker 1989). Since *R. sylvatica* and *Rana temporaria* tadpoles exhibit odour preferences for odorants (orange and citral) that had been injected into the egg during embryonic development (Hepper & Waldman 1992), the embryonic environment, which is primarily maternally determined, seems to be important for

olfactory learning of referents for later recognition abilities. However, *R. sylvatica* tadpoles also have been shown to discriminate unfamiliar paternal half-sibs from unfamiliar non-kin, demonstrating that a common maternal factor is not necessary for kin recognition (Cornell *et al.* 1989).

In studies that have demonstrated self-referent phenotype matching, it is unclear whether templates are imprinted upon by kinship labels consisting of polygenic interactions of phenotypic traits, of phenotypic traits encoded by specific recognition loci, or of both. (For further discussion see Appendix 1: Critical Review of the Evidence for and Interpretation of Self-Referent Phenotype Matching).

Kin Recognition Facilitated by Recognition Alleles

The idea that kin recognition may be facilitated by specific 'recognition alleles,' is contentious because of the hypothetical complexity of such a system. Recognition loci must be polymorphic enough for recognition alleles to reliably predict relatedness and must be expressed phenotypically in a manner that can be discriminated by conspecifics. Hamilton (1964b) and Dawkins (1976) suggested that any allele that somehow effected an identifiable phenotype that caused its bearer to favour those conspecifics that shared the phenotype might spread more quickly by natural selection than would other alleles. Such recognition alleles may therefore be expected to rapidly become fixed in a population and become therefore unreliable predictors of relatedness (Waldman 1987). However, additional pleiotropic effects, such as directing inbreeding avoidance or determining different disease susceptibilities, may maintain polymorphisms at loci for recognition alleles (Waldman 1987).

There are three distinct hypothetical mechanisms by which 'recognition alleles' may facilitate kin recognition which often get confused with one another.

- There may be one locus responsible for the both the kinship label and the cooperative responses (green beard hypothesis) (Dawkins 1976). There is little empirical support

for such a system and it is problematic because such a green beard gene would influence the behaviour of its bearer in its own interest, and would therefore not necessarily direct behaviours in favour in the bearer's kin (Alexander & Bargia 1978). Green beard genes might be selected for even at the expense of other genes in an intragenomic "tug of war" with other green beard genes influencing the bearer's behaviour with competing interests (Waldman 1987). Furthermore, green beard genes might be susceptible to other genes that produce the same phenotypic marker without the cooperative responses (Waldman 1987). Nonetheless, Keller & Ross (1998**)** found an allele in fire ants (*Solenopsis invicta*) that can be discriminated through olfactory cues and causes workers bearing that allele to kill queens which do not bear that allele (Keller & Ross 1998). However, a possible interpretation could be that the 'cooperative killing behaviour' exists even in the absence of the allele causing the recognition cues. The pronounced 'killing behaviour' in individuals carrying the alleged green beard allele studied by Keller and Ross (1998) may simply result from a phenotype that stands out more than other phenotypes produced by other alleles at that locus.

- There may be separate linked loci responsible for the kinship labels and the responses to such labels (Yamazaki *et al.* 1976). There is also no empirical support for such a system and it suffers from the same theoretical limitations as the green beard hypothesis.
- Most studies that provide evidence for the existence of recognition alleles suggest that there are both genetic and familiarity components to kin recognition. The most parsimonious mechanism is therefore that recognition alleles are responsible only for the kinship labels that are then discriminated based on phenotype matching. The discrimination of kinship labels and the associated altruistic behaviours are also

likely to be determined by independent mechanisms. This is supported by theoretical modelling based on the prisoner's dilemma game (Jansen & Baalen 2006). If recognition and altruistic cooperation are always inherited together, the dynamics are too unstable for the maintenance of cooperation, whereas if there is a more fluid association of altruistic traits with a recognition label, both are allowed to persist in weakly structured populations (Jansen & Baalen 2006).

Whether recognition alleles directly affect behavioural preferences, or simply code for phenotypic characters recognized, has not been established, and may be impossible to establish empirically (Blaustein 1983; Waldman 1987). Studies that provide empirical evidence consistent with the recognition allele hypothesis are also consistent with the phenotype matching hypothesis and vice versa. Even in studies in which test subjects reared in isolation or mixed rearing regimes discriminated kin from non-kin, the argument can always be made that an individual's own phenotypic traits are most pervasive in its environment and are imprinted upon by phenotype matching (Blaustein 1983; Waldman 1987). Because learned familiarity with the self is difficult to control for, it may be virtually impossible to rule out the role of phenotype matching in kin recognition (Blaustein 1983). However, whether the process of phenotype matching is involved or not, the possibility of recognition alleles that facilitate kin recognition remains and can be empirically tested.

Levels of polymorphisms necessary for accurate kinship assessment have been found in major histocompatibility complex (MHC) loci, which are understood to mainly function in facilitating self/non-self immune recognition (see chapter 3). Furthermore, the MHC has also been shown to contribute to individual odour profiles that can be discriminated by various taxa (see chapter 3). To date, the MHC has not been implicated in biasing behavioural preferences in any anuran species. In this thesis I examine *Xenopus laevis* tadpole MHC-type association preferences as well as kin association preferences at various developmental stages.

Chapter 3 : Adaptive Immunity and MHC-Biased Behaviour

In this chapter I discuss the role of the major histocompatibility complex (MHC) in adaptive immunity and the preferential treatment of conspecifics (MHC-biased behaviour). Both functions maintain and capitalize on the high genetic diversity found at the MHC in most vertebrate populations. In turn, vertebrate populations benefit, in terms of host/parasite interactions, from high genetic diversity - particularly at the MHC. MHC polymorphisms may be maintained by both pathogen mediated selection and sexual selection mechanisms. Avoidance of MHC-similar mating partners is likely to result in inbreeding avoidance and maintain locally and generationally diverse MHC genotypes in a population – which can reduce and vary disease susceptibility in and among offspring. In addition to immune fitness benefits, the ability to discriminate among conspecifics based on MHC-type may also have been selected for to facilitate kin discrimination. There are several hypothetical mechanisms by which MHC-type discrimination can be facilitated. Furthermore, the MHC has the potential to facilitate not only MHC-type discrimination, as found in a variety of controlled studies, but also to facilitate discrimination of genome-wide relatedness, and thereby kin recognition.

In this thesis, I study MHC-type discrimination not in the context of mate choice, but in association preferences of *Xenopus laevis* tadpoles. Tadpoles of many anuran species preferentially associate with kin, benefit from kin association in their growth and development, or both. I introduce *X. laevis* as a model organism for the study of adaptive immunity and MHC-biased behaviours as its immunogenetics is understood best among anurans and one can study MHC-type discrimination at varied developmental stages marked by changes in olfactory processing - from tadpoles through metamorphosis to adult frogs.

The Major Histocompatibility Complex (MHC)

The major histocompatibility complex (MHC) is a multigene family comprising loci that code for a number of different molecules, including the highly polymorphic classical histocompatibility molecules (Hughes & Hughes 1995; Hess & Edwards 2002; van den Berg & Rand 2003). MHC molecules bind broken-down protein fragments non-specifically and display them on cell surfaces. If the antigen displayed is foreign to the host, T-cells with receptors specific for that MHC/antigen complex will bind it and initiate a cascade of immunological events, known as the adaptive immune response (Klein & O'Huigin 1994; Potts *et al.* 1994; Hess & Edwards 2002).

Heritable histo(tissue)-compatibility was discovered in a series of tissue transplantation experiments (Little 1916; Bover 1927; Gorer 1936; Snell 1948). In these experiments, skin transplanted from a mouse of an inbred strain onto a mouse from another inbred strain was recognized as foreign and soon died; it was rejected (Little 1916). However, when the donor and the recipient mice were identical twins, grafts were not rejected (Bover 1927). A series of graft rejection experiments were conducted to find out which genes code for the proteins causing the graft rejection (Gorer 1936; Snell 1948; Du Pasquier & Chardonnens 1975). A gene complex that stood out in all these experiments appeared to be responsible for the graft rejections (Gorer 1936). When donor and host genes showed differences in this complex, rejection was very rapid (Gorer 1936; Snell 1948; Du Pasquier & Chardonnens 1975; Du Pasquier 2001). Because of the importance of these genes in graft rejection or acceptance, they were named histocompatibility genes (Snell 1948), and the gene complex came to be known as the major histocompatibility complex (MHC).

The main function of MHC molecules in the adaptive immune response is the control of antigen recognition by T lymphocytes (Parham & Ohta 1996). These T-cells, in turn, control cellular immunity and the B lymphocyte response (Parham & Ohta 1996). The classical histocompatibility molecules are cell surface glycoproteins that bind broken-down protein fragments with low specificity (Klein & O'Huigin 1994). Such MHC-protein

structures can be bound by T-cell receptors that are highly specific (Klein & O'Huigin 1994; Parham & Ohta 1996); each T-cell receptor is only capable of binding specific MHC-protein structures. Moreover, T-cell receptors are only able to recognize specific peptide antigen fragments when they are presented by MHC molecules (Lo *et al.* 1986; van den Berg & Rand 2003). Without MHC molecules, T-cells would not be able to recognize and bind antigens. Intact antigens need to be converted into a peptide(antigen)-MHC-complex and then be presented on the plasma membranes of antigen presenting cells (APC) to be recognized by T-cells (Edwards & Hedrick 1998). T-cell recognition of an MHC-protein structure triggers an immune response to the APC (Klein & O'Huigin 1994; Potts *et al.* 1994).

Rolf M. Zinkernagel and Peter C. Doherty were awarded the 1996 Nobel Prize in Physiology or Medicine for their discovery that the MHC restricted cellular immunity to viral infection. In their experiments, sensitized T-cells from a virus infected mouse would only destroy virus infected APC's from another mouse if it was MHC identical (Zinkernagel & Doherty 1974b, 1974a). This work demonstrated that for an immune response to be initiated, T-cells not only must recognize the MHC-presented foreign antigens as 'non-self', but must also recognize the presenting MHC molecule as 'self'. It is important to note that graft rejection due to T-cell recognition of foreign MHC molecules can still occur due to overlap of alloreactive T-cell repertoires between different MHC types (Sherman & Chattopadhyay 1993).

Each organism produces a repertoire of different T-cell receptors by gene rearrangement processes similar to those responsible for antibody diversity (Davis & Bjorkman 1988; Potts *et al.* 1994). Efficient 'non-self' recognition and self-tolerance is the result of intrathymic T-cell selection. First, during positive T-cell selection, cells having some specificity for the MHC molecules of the individual are screened and directed to differentiate into mature T-cells (Lo *et al.* 1986). Subsequently, during negative T-cell selection, cells that recognize MHC molecules bound to proteins originating from the organism's own cells are screened out and destroyed in the thymus before they are released into the blood stream (Lo

et al. 1986; Potts *et al.* 1994). As a result of this process, an organism's MHC type determines its T-cell receptor repertoire.

The ability of an organism to recognize a pathogen depends on that individual's T-cell repertoire, which is MHC dependent. If there were only a few MHC types in a population, pathogens would be more likely to evade recognition by the resultant T-cell receptors and successfully infect all individuals in the population. However, the genes encoding the MHC molecules are among the most polymorphic genes known in the animal kingdom (Du Pasquier *et al.* 1986, Borghans *et al.* 2004), making it very difficult for rapidly evolving pathogens to escape MHC-dependant immune recognition. The polymorphisms of the MHC are confined to the peptide binding region (PBR) of the major histocompatibility molecules (Zelano & Edwards 2002). They are 'genetic hot spots' in which non-synonymous mutations occur with remarkable frequency (Hughes & Nei 1988). The human MHC has 21 polymorphic loci with over 1000 identified alleles and up to 349 alleles described for a single locus (Robinson *et al.* 2000). MHC polymorphisms are so extensive that each individual has an identity, or 'self', defined by his or her particular set of MHC genes.

MHC alleles are co-dominant (both alleles at a locus are expressed) (Du Pasquier *et al.* 1989; Du Pasquier & Flajnik 1990; Flajnik 1996; Flajnik & Kasahara 2001) and MHC heterozygosity generally exceeds those predicted by neutrality (Edwards & Hedrick 1998; Ohta 1998). The diversity of T-cell repertoires among individuals in a population results in part from the heterozygous expression of MHC loci. Thus the MHC can function as an effective cellular self-marker or marker relatedness.

Selection for MHC Polymorphisms

Even though the polymorphisms of the MHC allow it to function as a 'self-marker' that facilitates self/non-self recognition in the immune system, how MHC polymorphisms are maintained has remained the subject of wide scale debate (Doherty & Zinkernagel 1975;

Takahata & Nei 1990; Hedrick 1992; Alberts & Ober 1993; Hughes & Hughes 1995; Potts & Slev 1995). Possible mechanisms that may select for MHC polymorphisms include pathogen driven selection mechanisms as well as sexual selection mechanisms, and these are not mutually exclusive.

Pathogen-Driven Frequency-Dependant Balancing Selection

Because of the benefits that the diversity of the MHC confers to pathogen-resistance, the genetic diversity of the MHC is widely attributed to pathogen-driven frequency-dependent balancing selection (rare allele advantage) (Takahata & Nei 1990; Hedrick 1992). According to the hypothesis, pathogens rapidly evolve to evade recognition from common MHC alleles in a population. As a result, rare MHC alleles may be more resistant to common pathogens, allowing them to increase in frequency until pathogens mutate to evade recognition resulting from those alleles. The time-lag nature of these antagonistic co-evolutionary responses could lead to the cycling of frequencies and fitness values of different MHC alleles within a population (Bernatchez & Landry 2003).

For pathogen-driven balancing selection to occur, infection must reduce host reproduction or survival and host genotypes must differ in their susceptibility (Little 2002). This hypothesis also requires that parasites are able to adapt to host genotypes (Penn *et al.* 2002). Such co-evolution may be difficult to demonstrate in practice as the genetic basis of host–pathogen interactions may be more complex than is represented by a simple gene-for-gene model (Woolhouse *et al.* 2002). For example, it has been suggested that resistance to pathogens may be a polygenic trait (Webster & Davies 2001). Even though computer simulations can explain the diversifying selection of the MHC by host-pathogen co-evolution (Takahata & Nei 1990; Satta *et al.* 1994; Borghans *et al.* 2004), the low selection intensity of host-pathogen co-evolution places severe restrictions on the possibility of measuring selection directly in vertebrate host populations (Satta *et al.* 1994). Measuring allele

frequency changes over time would not likely be possible if measurements occurred for less than 20 generations or with sample sizes of <5000 individuals (Satta *et al.* 1994).

Nonetheless, there is increasing evidence for host-parasite co-evolution that may contribute to the diversifying selection acting on MHC loci. People from West Africa bearing certain rare MHC alleles are more resistant to malaria infection than individuals that do not bear these MHC alleles (Hill *et al.* 1991). Lohm *et al.* (2002) found MHC-allele-specific resistance in Atlantic salmon (*Salmo salar* L.) to an infectious bacterium (*Aeromonas salmonicida*). Individuals bearing the more resistant MHC alleles were up to 1.5 times as resistant to the bacterium than siblings bearing the more susceptible MHC alleles (Lohm *et al.* 2002). In *Xenopus laevis,* certain MHC alleles confer a greater resistance to viral infection. Gantress *et al.* (2003) exposed outbred MHC heterozygous and two strains of inbred MHC homozygous (*ff*, *jj*) *X. laevis* frogs to frog virus 3. Frogs from the *jj* strain took twice as long to clear the viral infection than *ff* strain frogs or outbred frogs (Gantress *et al.* 2003).

The most compelling evidence for a rare-allele advantage comes from recent research in HIV patients (Trachtenberg & Funkhouser 2003; Scherer *et al.* 2004). Reclassification of MHC class I alleles into nine major supertypes based on their peptide binding properties revealed that rare MHC supertypes are associated with lower viral loads and slower disease progression than more common MHC supertypes (Trachtenberg & Funkhouser 2003). Further investigation into the T-cell responses of HIV patients revealed that both rare MHC alleles and MHC alleles associated with slow disease progression elicited detectable T-cell responses in greater proportions than in patients bearing more common MHC alleles and as MHC alleles associated with rapid disease progression respectively (Scherer *et al.* 2004). These results were also significant when reclassifying the MHC alleles into the major supertypes as in Trachtenberg *et al.* (2003) (Scherer *et al.* 2004). These findings suggest that individuals bearing rare MHC alleles are less likely to be infected with HIV strains that are pre-adapted to the MHC selected T-cell responses that they can make (Scherer *et al.* 2004).

Selection for a Heterozygote Advantage

Doherty and Zinkernagel (1975) hypothesized that MHC polymorphisms are maintained by selection for a heterozygote advantage. The co-dominant nature of MHC allele expression (Du Pasquier *et al.* 1989; Du Pasquier & Flajnik 1990; Flajnik 1996; Flajnik & Kasahara 2001) may enable MHC-heterozygotes to display a wider array of protein fragment epitopes than do MHC-homozygotes, positively selecting for a greater number of T-cell receptors in the thymus (Hughes & Hughes 1995). The greater number of T-cell receptors may in turn enhance immune responsiveness (Doherty & Zinkernagel 1975).

Hypothetically however, high levels of MHC allele diversity within individuals also may decrease T-cell repertoires by negative thymoid selection and thereby reduce immune responsiveness. During negative T-cell selection in the thymus, cross-reactivity of the different dimeric molecular expressions of MHC alleles (aa or bb homodimers or ab heterodimers) with the different sets of T-cells may reduce the number of T-cell receptors selected for in the thymus. Borghans *et al.* (2003) conducted mathematical modelling of the trade-off between maximizing the detection of foreign antigens and minimizing the loss of T cell clones due to self-tolerance induction during negative T-cell selection. Their model suggests that T-cell repertoire sizes will only begin to decrease at unrealistically high MHC diversities exceeding 1,500 different MHC molecules per individual (Borghans *et al.* 2003).

Computer simulation models suggest that pathogen-driven selection for heterozygotes can increase the coalescence time of allelic lineages which would result in the maintenance of MHC polymorphisms (Hughes & Nei 1988; Takahata & Nei 1990; Takahata *et al.* 1992; Satta *et al.* 1994; Hughes & Hughes 1995). However, others suggest that heterozygote advantage on its own is insufficient to explain the high population diversity of the MHC (Lewontin *et al.* 1978, Parham *et al* 1989, Wills 1991, De Boer *et al.* 2004). A computer model by De Boer *et al.* (2004) which assumes that the fitness of an individual is directly related to the properties of the MHC alleles it harbours did not support the hypothesis that

heterozygote advantage accounts for MHC diversity in a population. However, the fitness benefits of certain alleles may be entirely context/pathogen dependent. A heterozygote advantage is better understood as the resistance it may confer over a lifetime to multiple different pathogens.

Theoretically, a heterozygote advantage may be the result of either overdominance or dominance. If the expressed MHC heterodimer found in MHC heterozygotes confers resistance that neither of the homozygote haplotypes have, the heterozygote advantage is due to overdominance (McClelland *et al.* 2003). If the resistance of heterozygotes is better than the average of homozygotes, but not better than the most resistant homozygote haplotype, the heterozygote advantage is a consequence of the dominance of specific alleles that confer greater resistance to specific pathogens (Penn *et al.* 2002).

Heterozygosity at certain MHC loci is associated with reduced HIV progression (Carrington *et al.* 1999) and reduced Hepatitis B persistence (Thursz *et al.* 1997). However, in neither of these studies has the observed heterozygote advantage been disentangled from the dominance of more resistant alleles among differentially susceptible alleles. When exposing mice to *Salmonella* strains, heterozygotes were more resistant than the average of parental homozygotes, but were not more resistant than both parental homozygote haplotypes (Penn *et al.* 2002). The overall heterozygote advantage seemed to be conferred by the dominance of the more resistant MHC allele rather than by overdominance (Penn *et al.* 2002).

The dominance of more resistant MHC alleles also can provide a mechanistic basis for how MHC overdominance can occur during dual infections (McClelland *et al.* 2003). Heterozygote advantage due to overdominance was found in MHC-congenic mouse strains coinfected with *Salmonella enterica* and Theiler's murine encephalomyelitis virus (TMEV) where one haplotype was resistant to *Salmonella* and the other was resistant to TMEV (McClelland *et al.* 2003). During coinfection, the overall pathogen load of heterozygotes was

significantly lower than that of either of the parental homozygote haplotypes (McClelland *et al.* 2003). To my knowledge, this is the only empirical experimental evidence of MHC-heterozygote advantage due to overdominance. However, the overdominance observed in this study seems to be the result of specific resistances conferred by the different alleles. Long-term sequential infection studies may reveal if such heterozygote advantage is the result of having more alleles that can detect more epitopes or if the MHC heterodimers also confer overdominant immunity that neither of the MHC homodimers do.

MHC Polymorphisms Maintained by Sexual Selection

Strong evidence suggests that MHC polymorphisms are driven by sexual selection (Wedekind & Penn 2000). Numerous studies in various taxa have demonstrated that individuals discriminate mating partners based on MHC type (Yamazaki *et al.* 1976; Egid & Brown 1989; Potts *et al.* 1991; Wedekind *et al.* 1995; Ober *et al.* 1997; Wedekind & Furi 1997; Eklund 1998; Penn & Potts 1998a; Landry *et al.* 2001; Reusch *et al.* 2001; Zelano Edwards 2002; Aechlimann *et al.* 2003; Freeman-Gallant *et al.* 2003; Olsson *et al.* 2003; Milinski *et al.* 2005; Richardson *et al.* 2005). MHC-biased mating may be a function of how well MHC types of mating partners complement each other to optimize genetic diversity and the range of offspring disease resistance or it may be condition-dependent, favouring certain MHC-types that confer specific resistances to relevant pathogens. I will discuss how MHC polymorphisms may be maintained by complementary mate choice that favours either dissimilar MHC types (MHC-disassortative mating) or MHC allelic diversities that are complementary to produce the optimal MHC diversity in the resultant offspring (optimal MHC-diversity). I also will discuss how the MHC can influence condition-dependent mate choice that favours alleles that are more resistant to the pathogens in the environment at the time.

The different MHC-linked mating strategies are context-dependent and can vary between species based on how likely they are to mate with related individuals by chance or

on how rapidly pathogens change in their ecological environment (Reusch *et al.* 2001). Mating strategies may even vary within species as they can occupy different habitats that may expose them to changing selection pressures. Computer simulations predict that condition-dependent mate choice is likely to replace random mate choice and that complement-dependent mate choice will be selected for without going to fixation in populations that otherwise exhibit random or condition-dependent mate choice (Howard & Lively 2004). These results suggest that the different mate choice strategies have similar fitness benefits and can co-occur (Howard & Lively 2004).

Selection Due to MHC-Disassortative Mating

MHC-disassortative mating may contribute to diversifying selection acting on MHC loci (Hedrick 1992; Penn & Potts 1999). Experiments conducted on a variety of species, including mice (Yamazaki *et al.* 1976; Egid & Brown 1989; Potts *et al.* 1991; Penn & Potts 1998a), lizards (Olsson *et al.* 2003), sparrows (Freeman-Gallant *et al.* 2003), salmon (Landry *et al.* 2001), and humans (Wedekind *et al.* 1995; Ober *et al.* 1997; Wedekind & Furi 1997) indicate that individuals prefer to mate with others that have dissimilar MHC genes. These mating preferences have been observed in laboratory mate choice experiments (Yamazaki *et al.* 1976; Yamazaki *et al.* 1988; Egid & Brown 1989; Potts *et al.* 1991; Penn & Potts 1998a) as well as in field studies that compared MHC similarities of mating partners to those expected under random mating (Ober *et al.* 1997; Landry *et al.* 2001; Freeman-Gallant *et al.* 2003; Olsson *et al.* 2003).

Male mice (*Mus domesticus*) were the first vertebrates shown to have MHC-disassortative mating preferences in laboratory enclosures in which male mice mated first with the MHC-dissimilar potential mating partner when given a choice of two potential mating partners that differed only in the MHC (Yamazaki *et al.* 1976, 1988). Female mice in oestrus were subsequently shown to have similar mating preferences in an improved testing apparatus in which the potential male mating partners could not interact; they were tethered

in separate compartments within the enclosures (Egid & Brown 1989). MHC-disassortative mating preferences also were observed in large enclosures that contained 24 to 31 mice each (Potts *et al.* 1991; Penn & Potts 1998c). These 'semi-natural' mouse populations were derived from inbred strains with known MHC haplotypes crossed with outbred mice. Potts *et al.* (1991) found that mating preferences in these 'semi-natural' mouse populations resulted in 27% fewer MHC-homozygous offspring than expected under random mating.

More recent studies on natural populations of Swedish sand lizards (*Lacerta agilis*) (Olsson *et al.* 2003), Savannah sparrows (*Passerculus sandwichensis*) (Freeman-Gallant *et al.* 2003), Altantic salmon (*Salmo salar*) (Landry *et al.* 2001) and humans (Ober *et al.* 1997) have demonstrated that mating partners tend to be more MHC dissimilar than expected from random mating. Female Savannah sparrow yearlings not only avoided pairing with MHC-similar males but MHC similarity between mates also predicted the occurrence of extra-pair young in first broods (Freeman-Gallant *et al.* 2003). In humans, MHC similarities between spouses among 411 Hutterite couples were less than expected under non-random mating patterns with respect to colony lineages (Ober *et al.* 1997). Among couples who did match for a haplotype, most matched haplotypes were paternally inherited, with maternally inherited MHC-haplotype similarities being predominantly avoided in human mate choice (Ober *et al.* 1997). This suggests that the maternally inherited MHC haplotypes are primarily imprinted upon early in development, perhaps in the embryonic environment, as has been found in kin recognition studies in amphibians (Waldman 1981; Blaustein & O'Hara 1982b; Hepper & Waldman 1992).

The MHC also has been implicated in human mate choice by experiments that examined odour and facial preferences. Women scored the odours of T-shirts worn by men who were more MHC-dissimilar as more pleasant (Wedekind *et al.* 1995; Wedekind & Furi 1997; Jacob *et al.* 2002; Santos *et al.* 2005). Male odour preferences have been found to be similar (Wedekind & Furi 1997; Thornhill *et al.* 2003). These odour preferences may be correlated with mating preferences as men and women were also reminded of their own

mate/ex-mate when smelling a T-shirt whose wearer they had significantly fewer MHC-alleles in common with than expected by chance (Wedekind *et al.* 1995; Wedekind & Furi 1997). In contrast, Thornhill *et al.* (2003) found that odour preferences of ovulating women were independent of MHC similarity, but instead correlated with the MHC-heterozygosity of the T-shirt wearers. Similarly, women rated the faces of more MHC heterozygous males as more attractive (Roberts *et al.* 2005). These preferences were also independent of the degree of MHC similarity between the men and women raters (Roberts *et al.* 2005).

SELECTION FOR MHC-DISASSORTATIVE MATING

Because higher proportions of MHC heterozygotes result from MHC-based disassortative mating than from random mating, the selective forces that result in MHC-based disassortative mating may account for the maintenance of MHC polymorphisms. Selection for MHC-disassortative mating preferences may be pathogen driven. As none of the potential mechanisms of maintaining MHC polymorphisms discussed above are mutually exclusive, MHC-disassortative mating may serve not only to maintain MHC polymorphisms, but may have been selected for by the enhanced fitness it provides the offspring (Ihara & Feldman 2003). MHC-disassortative mating may have been selected to optimize the offspring's resistance to infectious diseases by ensuring any fitness benefit that MHC heterozygosity may confer to progeny (heterozygote advantage hypothesis) (Potts & Wakeland 1990; Penn 2002) or by providing a 'moving target' of extant MHC polymorphisms for rapidly evolving parasites that escape MHC-dependent immune recognition (Red Queen hypothesis) (Penn & Potts 1999). Alternatively, because the ability to discriminate based on the MHC may facilitate kin discrimination, MHC-disassortative mating may have been selected for to avoid the deleterious effects of inbreeding by facilitating non-kin mating (inbreeding avoidance hypothesis) (Brown & Eklund 1994). Again, none of these are mutually exclusive.

HETEROZYGOTE ADVANTAGE

If there is a heterozygote advantage resulting from the co-dominant expression of MHC genes, natural selection may favour the evolution of MHC-based disassortative mating preferences. Such behaviour would increase reproductive fitness by ensuring increased immunocompetence resulting from the MHC heterozygote advantage in progeny (Potts *et al.* 1994). This is supported by the work of Landry *et al.* (2001), who found that Atlantic salmon choose their mates to increase the heterozygosity of their offspring at the MHC, even after controlling for inbreeding avoidance (genetic relatedness of the preferred mates supported a random mating scheme). Ruelicke *et al.* (1998) found that MHV (mouse hepatitis virus) infected mice produced more MHC-heterozygous embryos than sham-infected ones, which suggests that MHC heterozygosity confers greater disease resistance that can be selected for *in utero*.

MOVING TARGET / RED QUEEN

MHC-disassortative mating may provide a 'moving target' of changing allelic combinations from generation to generation to prevent pathogens from evading immune recognition. This 'moving target' or 'Red Queen' (continuously running while staying in the same spot) hypothesis assumes that the disease resistances conferred by different MHC alleles are under frequency-dependent selection in co-evolution with rapidly evolving pathogens (Penn & Potts 1999). If parasites adapt to a host's MHC genotype, then MHC-disassortative mating preferences may function to obtain MHC allele combinations for offspring that are different from the parents' (Penn & Potts 1999; Penn 2002; Thornhill *et al.* 2003). Pathogens adapted to parental immune systems are then less adapted to offspring immune systems because they have different T-cell repertoires (Penn & Potts 1999). An offspring's immune system would therefore be more likely to recognize pathogens that have evaded either parent's immune recognition. MHC-disassortative mating preference may function to produce offspring that are MHC-dissimilar from their parents rather than being

heterozygote *per se* (Penn & Potts 1999). These mating preferences could thereby also slow the rate at which pathogens adapt to a host's MHC genotype (Penn & Potts 1999).

INBREEDING AVOIDANCE

MHC-disassortative mating may select for the reproductive success and fitness associated with inbreeding avoidance. The inbreeding avoidance hypothesis suggests that MHC-disassortative mating is similar to other genetic incompatibility systems that function to reduce inbreeding (e.g. plant or tunicate histocompatibility systems) (Bernatchez & Landry 2003). Since closely related individuals are similar at the MHC (Potts *et al.* 1994), the ability to discriminate based on the MHC may function to facilitate kin recognition. MHC-disassortative mating may have been selected for to facilitate inbreeding avoidance, ensuring not only MHC heterozygosity, but also enhancing genome wide heterozygosity (Penn & Potts 1999; Penn 2002). The main genetic consequences of close inbreeding are an overall increase of homozygosity, which can reduce fitness due to increased expression of recessive deleterious mutations, and loss of any heterozygote advantage (Charlesworth & Charlesworth 1987). For species that are at high risk of inbreeding, such as some species of birds, amphibians, and fish which undertake migrations in early life but show a strong philopatry to the natal site for breeding, inbreeding avoidance may be very important and may require a genetically based kin recognition system (Jordan & Bruford 1998; Penn & Potts 1999).

IS MHC-DISASSORTATIVE MATING A FUNCTION OF THE MHC OR OF GENOME WIDE RELATEDNESS?

Hughes and Hughes (1995) point out that the avoidance of mating with MHC-similar individuals may be the consequence of inbreeding avoidance rather than MHC-disassortative mating *per se*. MHC-associated cues may be among many associated with kinship, all of which may be mediated by the ability to discriminate the familiar from the unfamiliar (Hughes & Hughes 1995). Indeed, there is substantial evidence for this. Cross-fostering by

MHC-different parents results in reversed MHC-associated mating preferences in both male (Yamazaki *et al.* 1988) and female mice (Penn & Potts 1998a). Cross-fostered mice prefer to mate with MHC-identical mice over mice bearing the MHC type of foster parents. These findings suggests that MHC-linked odour discrimination in mice is determined neither by a direct genetic mechanism (recognition alleles) nor by the use of self-referent phenotype matching, but rather by familiarity, and more specifically, familial imprinting (Penn & Potts 1998a). In juvenile Arctic charr (*Salvelinus alpinus*), preferences for the odours of MHC-identical siblings over MHC-different siblings (Olsen *et al.* 1998, 2002) were not observed when the test fish were isolated since fertilization (Olsen *et al.* 2002). These findings suggest that the ability to discriminate MHC-linked odours is learned (Olsen *et al.* 2002).

Non-MHC-linked genes also may contribute to cues that carry information about individuality or relatedness. Even though juvenile Arctic charr can discriminate between the odours of MHC-identical and MHC-different siblings, no preferences between MHC-identical non-siblings and MHC-different siblings were observed (Olsen *et al.* 2002). These results suggest that the MHC as well as other genes determine the schooling preferences (Olsen *et al.* 2002). In mice, the ability to distinguish between the odours of two strains disappeared after randomizing the genomic background of the stimulus mice (Carroll *et al.* 2002). Mice also have been shown to discriminate odours of conspecifics based on differences in genes on sex chromosomes (Yamazaki *et al.* 1986a). Also, MHC-differences alone do not stimulate competitive scent marking behaviour in mice whereas genome-wide differences do (Hurst *et al.* 2005). All of these findings provide evidence that other genes are involved in odourtype recognition that may influence subsequent mating behaviour.

Most studies on MHC-disassortative mating cannot rule out the possibility that MHC-linked genes, rather than the MHC itself, are responsible for MHC-type discriminations. However, there are studies that show that differences in specific MHC loci can be discriminated. Mice are able to distinguish the odours of conspecifics that differ only by a deletion mutation in a specific MHC locus (Penn & Potts 1998c), by a point mutation in a

specific MHC locus (Yamazaki *et al.* 1982), or between isogenic mice that can or cannot express a specific MHC locus (Bard *et al.* 2000). Outbreeding of inbred mouse strains to create more background genetic variation did not decrease the ability of mice to discriminate odours of conspecifics by MHC type (Yamazaki *et al.* 1994; Yamazaki & Beauchamp 2005). Furthermore, rats can discriminate the odours of urine of genetically identical mice that were either injected with recombinant soluble MHC molecules or a sham injection (Janssen *et al.* 2001).

Nonetheless, observations of MHC-associated preferences along with certain adaptive consequences cannot be interpreted teleonomically as adaptations. Suggesting that MHC-disassortative mating may select for the maintenance of MHC polymorphisms does not imply that the behaviour was selected for by the benefits of diverse MHC polymorphisms directly. Suggesting that MHC-disassortative mating may increase the fitness of offspring does not imply that the ability to discriminate conspecifics based on MHC-linked traits was selected for to facilitate MHC-disassortative mating, though it cannot be discounted. The MHC simply may be a significant contributor to the scent of individuality because it is so polymorphic and is involved in the processing of a wide range of antigens. The resulting ability to distinguish MHC-linked traits may be independent of behavioural preferences. Behavioural biases may have evolved to capitalize on inherent MHC-linked traits and MHC-disassortative mating preferences may have been selected for by fitness benefits related to inbreeding avoidance or the MHC or both. Either way, MHC-disassortative mating preferences can reduce genetic homozygosity and maintain MHC polymorphisms (Hedrick 1992).

Selection for Optimal MHC-Diversity in Offspring

Extensive research on stickleback fish (*Gasterosteus acaleatus*) suggests that MHC polymorphisms also may be maintained by mating preferences that optimize the diversity of MHC alleles across multiple loci. Sticklebacks possess up to six recently duplicated MHC

loci with shared sets of potential alleles (Reusch *et al.* 2001). Since individuals may have duplicated copies of specific alleles on separate loci, the number of distinct MHC alleles may vary up to 12. Gravid female sticklebacks preferred the odour of males with more MHC alleles rather than the odours of MHC dissimilar males *per se* (Reusch *et al.* 2001). In a flow channel apparatus, these odour preferences reliably predict mate choice (Milinski *et al.* 2005). The observed mating preferences result in greater MHC allele diversity within offspring, providing a mate choice mechanism distinct from MHC-disassortative mating that can maintain MHC polymorphisms (Bakker & Zbinden 2001; Reusch *et al.* 2001). Further research found that this preference is reversed in females that already have exceptionally high MHC diversity. Females with high MHC diversity chose mates that produced offspring with intermediate MHC allele diversity (Milinski 2003; Milinski *et al.* 2005). Such intermediate MHC allele diversity also has been shown to confer optimal immunocompetence in stickleback fish as they are associated with minimal parasite loads when compared to individuals with maximal or minimal MHC allele diversities (Wegner *et al.* 2003a, 2003b; Kurtz *et al.* 2004). The reduced immunocompetence of individuals with too much MHC-diversity is postulated to be a consequence of both an increased risk of autoimmunity as well as too much negative thymoid selection on an individual's T-cell repertoire (Penn & Potts 1999; Bakker & Zbinden 2001; Wegner *et al.* 2003b).

Similar MHC-associated mating preferences also have been observed in the Seychelles Warbler (*Acrocephalus sechellensis*) in which extra-pair paternity (EPP) is more likely when the social mate has low MHC diversity (Richardson *et al.* 2005). Richardson *et al.* (2005) also found that the MHC-diversity of extra-pair males is significantly greater than that of the cuckolded males.

Selection for Specific Alleles that are Associated with Increased Immunocompetence

Hamilton and Zuk (1982) hypothesized that the monitoring of traits that indicate health and vigour may facilitate the assessment of the genetic quality of potential mates. MHC-

associated mate choice may in certain contexts be a function of specific MHC-alleles that confer more resistance to environmental pathogens and thereby result in increased health and vigor (Zelano & Edwards 2002). Since hosts fight off disease via resistance to particular pathogens, resistance to other pathogens may be lowered (Hamilton & Zuk 1982). Changes in pathogens over evolutionary time in turn change which MHC genes confer the best resistance (Westneat & Birkhead 1998). In concordance with specific MHC-alleles that may confer increased resistance to specific pathogens, studies in mice (Eklund 1998), Swedish sand lizards (*Lacerta agilis*; Olsson et al. 2005) and in the great snipe (*Gallinago media*; Ekblom et al. 2004) have demonstrated that certain MHC alleles are favoured in mate choice regardless of similarity. Olsson et al. (2005) also found that individual sand lizards that bear the preferred MHC allele have fewer ectoparasites under increasing physiological stress and are more successful at mate acquisition and mate guarding.

The Influence of the MHC on Fertilization and Gestation

Besides sexual selection mechanisms, the MHC also has been shown to influence fertilization success and gestational success. Skarstein *et al.* (2005) found that a particular MHC allele was associated with increased fertilization success in the Arctic charr (Skarstein *et al.* 2005). Studies on humans and rodents demonstrate that foetal loss rates are greater in pregnancies from MHC-similar gametes (Ober 1992; Apanius *et al.* 1997; Ober 1998; Rűlicke *et al.* 1998; Ober 1999; Penn & Potts 1999). Furthermore, the chemosensory recognition of MHC types has been shown to affect the reproductive hormonal status of pregnant females (Yamazaki *et al.* 1983b, 1986b). Pregnant female mice exposed to mice that differed at the MHC to the impregnating male mice are more likely to undergo pregnancy-block (Bruce effect) than if they were exposed to mice that are MHC-identical to the impregnating male mice (Yamazaki *et al.* 1983b, 1986b). Conversely, the reproductive hormonal status of females has been shown to influence MHC-biased association preferences. While female mice demonstrate MHC-disassortative association preferences when in eostrus, female mice in meteostrus preferentially associate with MHC-identical males (Egid & Brown

1989). Similarly, human preferences for T-shirt odours from MHC-dissimilar individuals are reversed in women using contraceptive pills (Wedekind *et al.* 1995; Wedekind & Furi 1997), which endocrinologically mimics pregnancy.

MHC-Discrimination and Kin Recognition in Varied Social Contexts

Even though MHC-biased mate choice is the only behavioural context that has direct implications to the evolution of the MHC itself, the underlying mechanisms of MHC-linked discrimination may facilitate kin recognition in other social contexts (Penn & Potts 1999). Female house mice in 'semi-natural' population enclosures prefer MHC-similar communal nesting partners to rear their offspring (Manning *et al.* 1992), though studies by Ehmann and Scott (2001) could find no evidence that females prefer MHC-similar females as communal nesting partners. Female mice are also more likely to retrieve pups that have the same MHC as their own offspring (Yamazaki *et al.* 2000). However, pups preferred foster mother MHC-type odours over maternal MHC-type odours (Yamazaki *et al.* 2000). The Malagasy giant jumping rat (*Hypogeomys antimena*), which is obligately monogamous, appears to exhibit no MHC-dependent mating preferences except for when a new male is chosen by a territory-holding female, in which case MHC–similar mates are preferred (Sommer 2005).

Olsen et al. (1998) examined MHC-linked association preferences in juvenile Arctic charr (*Salvelinus alpinus*) among siblings, thereby controlling for genetic variance elsewhere in the genome. Although significant associative preferences for MHC-identical siblings over MHC-different siblings were reported in juvenile Arctic charr (Olsen *et al.* 1998), the results are problematic. Aside from the small sample size tested (n = 5), control subjects showed biased side preferences. These preferences served as a baseline with which subjects' subsequent responses were compared, which effectively inflated those measures. Nonetheless, Rajakaruna *et al.* (2006) found similar association preferences for MHC-similar siblings over MHC-dissimilar siblings in sibships of Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*).

As with inbreeding avoidance, MHC genes may be used to discriminate kin from non-kin. The ability to use the MHC-linked cues to identify kin may facilitate nepotistic interactions in a variety of social contexts. Such interactions may at times be associated with negative direct fitness consequences but are counterbalanced by increased inclusive fitness if they are disproportionately associated with close relatives (Hamilton 1964). Species that aggregate may be good model organisms to test biased social interactions among the most closely related of kin, siblings, as well as the role of the MHC in kin-recognition. The strongest functional link between histocompatibility and kin recognition has been established in marine chordates (see De Tomaso 2006). Colonies of the tunicate, *Botryllus schlosseri,* fuse only if they share the same allele at a single fusability locus (Scofield *et al.* 1982). The same locus also facilitates inbreeding avoidance by preventing fertilization of gametes sharing alleles (Scofield *et al.* 1982) and the settling of larvae near colonies that share fusability alleles (Grosberg & Quinn 1986).

How is MHC-Type Discrimination Facilitated?

Not only is the MHC in most vertebrate species polymorphic enough for the discrimination of genetic relatedness, but a growing body of research suggests that there are several mechanisms by which MHC polymorphisms can result in trait polymorphisms sufficient for MHC-type discrimination.

Odour

It is not clear how individuals are able to discriminate among conspecifics based on the MHC or MHC-linked traits. Most studies seem to indicate that olfaction plays an important role in MHC-biased discrimination and urine has been suggested to act as a carrier for these olfactory cues in mice and rats (Ehman & Scott 2001). Other carriers may be important as MHC odourtypes are also discernible in blood (Yamazaki *et al.* 1999). Urinary odours of mice are derived from preputial gland secretions; the removal of these glands results in the

loss of mouse odour preferences between congenic strains of mice that differ at the MHC (Ninomiya & Brown 1995). It is, however, unclear if this is the result of a loss in the ability to detect MHC-associated odour differences or of the loss of other pheromone cues that may trigger sex-biased responses.

Laboratory mice can be trained to distinguish the urinary odours of congenic strains that differ only at a single MHC locus (Yamazaki *et al.* 1979, 1982, 1983a; Yamaguchi *et al.* 1981). These MHC-associated odourtypes can be discriminated in mice as young as 1 day (Yamazaki *et al.* 1992). Mice even can be trained to discriminate between genetically identical pregnant females carrying 9 to 18 day-old foetuses of different MHC types (Beauchamp *et al.* 1995). The foetal odourtype signal remains for a substantial time after the foetuses have been born (Beauchamp & Yamazaki 2005). Untrained rats (Brown *et al.* 1987; Singh *et al.* 1987) and wild-derived house mice (Penn & Potts 1998c) can also distinguish the smell of urine samples of donors that differ only at an MHC locus in habituation/dishabituation experiments.

MHC-type discrimination can sometimes occur across species barriers as both rats (Beauchamp *et al.* 1985) and humans (Gilbert *et al.* 1986) can be trained to distinguish the urinary scents of MHC congenic mice. Even an electronic nose, consisting of an array of chemophysical detectors made up of quartz microbalances and semiconducting metal-oxide sensors that change frequency or conductivity upon binding of very small numbers of individual molecules present in gas or solution, is able to distinguish the odourtypes of MHC congenic mouse strains that differ only at a single MHC locus (Montag *et al.* 2001).

It remains unclear how MHC genes or closely linked loci influence an individual's odour (Penn 2002). There are several hypotheses that have been suggested to explain how the MHC influences the body odour of an individual:

- Since MHC molecules occur in solution dissociated from cells and are excreted in large amounts in urine and in sweat, fragments of MHC molecules may function as

odorants (MHC molecule hypothesis) (Singh *et al.* 1987, 1988; Wedekind & Penn 2000). This is, however, unlikely because MHC molecules are large non-volatile proteins that can be denatured without destroying the distinguishability of MHC-mediated odours by mice (Singer *et al.* 1993; Wedekind & Penn 2000).

- MHC molecules may carry volatile aromatics (the carrier hypothesis) (Singer *et al.* 1997; Pearse-Pratt *et al.* 1998; Singh 1998). Trained mice are able to distinguish between different MHC mouse strains based on volatile urine fractions from anion exchange chromatography and diethyl ether extracts (Singer *et al.* 1997). Based on gas chromatographic data analyses, the differences in the odourtypes seem to result from differing proportions of volatile chemicals characteristic of each MHC type (Singer *et al.* 1997).
- The MHC may influence odour indirectly by shaping an individual's set of commensal microorganism populations (microflora hypothesis) (Yamazaki *et al.* 1990; Brown 1995; Penn & Potts 1998b; Wedekind & Penn 2000). Yamazaki *et al.* (1990) found that mice can distinguish between the urines of MHC-congenic mice reared in germfree conditions as readily as MHC-congenic mice reared in conventionally maintained conditions. However, in contrast, Schellinck *et al.* (1995) found that rats that could be trained to distinguish the odours of two congenic MHC-different mouse strains could not do so if the mice had been reared and housed under germfree conditions.
- The MHC-PBR determined pool of peptides that can serve as ligands for MHC molecules (Rammensee *et al.* 1999) may determine individual odour profiles (peptide hypothesis) (Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005). Peptide ligands of nine amino acids (9-mers) are typically used in MHC presentation (Rammensee *et al.* 1999; Burroughs *et al.* 2004; Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005).

> Considering the number of distinct 9-mers that are possible in the human proteome, Burroughs *et al.* (2004) calculated that the probability of a foreign peptide being identical to a self peptide to be about 0.2%. These results indicate that these small subunits carry sufficient information for self/non-self discrimination (Burroughs *et al.* 2004). When exposing pregnant mice to synthesized 9-mers based on known MHC peptide ligands of either familiar or unfamiliar MHC types added to familiar urine, Leinders-Zufall *et al.* (2004) found that mice were more likely to undergo pregnancy block in response to the 9-mers based on the peptide ligands of the unfamiliar MHC type. Similarly, Milinski *et al.* (2005) were able to predictably modify mate choice decisions of female sticklebacks by adding different combinations of the synthetic 9-mer peptides. Not only did they demonstrate that the peptide ligands serve as cues of MHC allele diversity, but also that the specific amino acid residues that are the binding sites of MHC molecules are responsible for the perception of the MHC ligands (Milinski *et al.* 2005).

One or any combination of these hypotheses may be correct, but the understanding of how exactly MHC genes influence differences in odours is still progressing (Penn & Potts 1998; Penn 2002).

Chemical Nature of MHC-Associated Chemosignals

There may be different types of chemosignals that can facilitate MHC-linked discrimination in different behavioural contexts (Leinders-Zufall *et al.* 2004; Brennan & Binns 2005). The previously mentioned study by Singer *et al.* (1997) suggests that MHC-linked odorants are volatile. However, Nevison *et al.* (2003) found that mice seem to lose the ability to recognize the urine of conspecifics when the urine was covered by a porous nitrocellulose sheet to which proteins bind thereby allowing for the release of volatiles but not non-volatiles. Individual odours therefore seem to be carried by non-volatile proteins, rather than by volatiles. These contradictory findings may be the result of different

behavioural contexts in which the experiments were conducted. In the study by Singer *et al.* (1997) mice were motivated by reward to express discriminatory behaviours. In the Nevison *et al.* (2003) study, the composition of the urine itself was not manipulated and the ability to discriminate the urine was determined by scent marking responses rather than by reward training.

Olfactory Processing of MHC-Linked Chemosignals

Not only is there evidence for the involvement of both volatiles and non-volatiles in odourtype discrimination, but there is also evidence for the involvement of different parts of the brain in the processing of the different types of cues that can convey similar information. The primary olfactory organs are responsible for processing volatile odours, whereas the functionally and anatomically distinct vomeronasal organ (VNO) (Bargmann 1997) is understood to be primarily involved in the detection of non-volatile pheromones, which transmit specific information of social or reproductive status among conspecifics and elicit behavioural and neuroendocrine responses (Bertmar 1981; Bargmann 1997; Hegde 2003).

Surgical removal of the VNO in mice does not impair their ability to be trained to discriminate between congenic strains of mice that differ only at the MHC (Wysocki *et al.* 2004; Yamazaki & Beauchamp 2005) nor the preference of male for female urine samples supplemented with MHC class I peptide mixtures specific for a different haplotype over the same urine samples supplemented with MHC class I peptide mixtures specific for the male's own haplotype (Spehr *et al.* 2006). These results suggest that the primary olfactory system is involved in MHC odourtype discrimination. This is further supported by the differential neuronal activation patterns in the main olfactory bulbs of mice exposed to MHC-differing congenic mouse strains (Schaefer *et al.* 2001, 2002; Spehr *et al.* 2006). However, *in vitro* preparations of intact mouse VNOs have different excitatory responses to different sets of non-volatile MHC peptide ligands depending on the MHC-type of the mouse from which the VNO slice preparation was taken (Leinders-Zufall *et al.* 2004). Even though the VNO and

non-volatile scent cues are not necessary for MHC odourtype discrimination when animals are trained to make this discrimination, they may be involved in mediating natural behavioural responses to odourtypes (Leinders-Zufall *et al.* 2004; Yamazaki & Beauchamp 2005). Clearly there are redundant yet distinct mechanisms for the discrimination among disparate sets of MHC-peptides or MHC molecules within the main and accessory olfactory systems in mice (Olson *et al.* 2006; Spehr *et al.* 2006).

Olfactory Receptors

MHC-linked olfactory receptor (OR) genes may encode olfactory receptors involved in the recognition of MHC-dependent or individual-specific odours (Eklund *et al.* 2000; Ziegler *et al.* 2000; Younger *et al.* 2001; Amadou *et al.* 2003; Bonneaud *et al.* 2004). Amadou et al. (2003) identified 59 polymorphic olfactory receptor (OR) loci that are part of the extended MHC region in humans and co-duplicate with MHC genes. Ziegler *et al.* (2000) found the OR genes linked to MHC loci in humans are polymorphic. Findings that MHC-linked OR loci share duplication with MHC loci, have duplicated extensively and are polymorphic highlight questions about reciprocal influences acting on the dynamics and evolution of the MHC and MHC-linked OR loci (Amadou *et al.* 2003). MHC-linked OR loci may therefore play a role in MHC-dependent mating preferences (Aeschlimann *et al.* 2003; Bonneaud *et al.* 2004).

The VNO expresses specific pheromone receptors that might specifically detect MHC-mediated odourants (Loconto *et al.* 2003; Leinders-Zufall *et al.* 2004). Of the known MHC-linked OR loci, two belong to the vomeronasal 1 (V1R) family (Ehlers *et al.* 2000), which are coexpressed with G-protein subunits and are restricted to the luminal VN epithelium (Hegde 2003). A second family of vomeronasal receptors (V2R) are co-expressed with different G-protein subunits and are restricted to the basal VN epithelium (Hegde 2003). V2Rs differ from the V1Rs by the presence of large N-terminal domains (Leinders-Zufall *et al.* 2004). Unlike V1Rs, V2Rs are co-expressed with non-classical MHC (class-Ib) molecules, that are

unlinked to the classical MHC, at the cell surface of vomeronasal sensory neurons (Ishii *et al.* 2003; Leinders-Zufall *et al.* 2004) in the basal layer of the VNO (Ishii *et al.* 2003; Loconto *et al.* 2003). Association with MHC molecules seems to be essential for the expression of V2Rs on dendrites of the vomeronasal sensory neurons (Loconto *et al.* 2003). Leinders-Zufall *et al.* (2004) postulated that the sequence-specific recognition of peptides derived from classical MHC presentation may be achieved by the N-terminal domain of certain V2R receptors (or receptor combinations), whereas the non-classsical MHC-class Ib molecules may serve as a general presentation device. Limitations in MHC-class Ib polymorphisms could be compensated by the combinatorial expression with polymorphic V2Rs (Hegde 2003).

The interactions of potential genes involved in MHC-odourtype determination provide a hypothetical mechanism by which the discrimination not only of MHC similarity but also genome wide relatedness may be facilitated by the MHC. Ultimately, discrimination may depend on peptides originating from presentation by classical MHC molecules, MHC-linked V1Rs, V2Rs, and non-classical MHC molecules whose loci are dispersed throughout the genome and may mirror genome wide-relatedness. Furthermore, the diversity of MHC-bound 9mer-peptide ligands originating from endogenous proteins will be determined by genome-wide genetics.

Adaptive Immunity and MHC-Biased Behaviours in *Xenopus laevis*

MHC-biased behaviours have not yet been investigated in *Xenopus laevis*, or any other amphibian species for that matter. Amphibians in general are excellent model organisms for the study of socially biased behaviours due to their complex life history as well as the fecundity of many amphibian species. Amphibians can be studied as larvae or as adults, in aquatic or terrestrial habitats, in various contexts that give us insight into the development of their behaviours that may be correlated with changes in ecological niche, anatomy, physiology or gene expression. *X. laevis* is particularly well suited to studies on the MHC and/or olfaction, as its MHC and its olfactory organs have been described extensively.

The Xenopus MHC

The MHC of *Xenopus* has often been referred to as the XLA, for the *Xenopus* leukocyte antigen system (Du Pasquier *et al.* 1989), but for general accessibility I continue to refer to the XLA as the *Xenopus* MHC.

Among the polymorphic classical histocompatibility molecules are the MHC-class Ia and the MHC-class II molecules, as defined in mammals (Nonaka *et al.* 1997a,. 1997b; Flajnik & Kasahara 2001). MHC-class Ia molecules bind endogenously derived peptides, mainly from viral proteins degraded intracellularly (Salter-Cid *et al.* 1998; Bernatchez & Landry 2003), and are expressed on the surfaces of all adult *Xenopus* cells with their highest expression on haemopoietic cells (Flajnik *et al.* 1990). In contrast, MHC-class II molecules bind exogenously derived peptides, extracellularly produced from mainly bacterial proteins (Bernatchez & Landry 2003), and are expressed on only a limited range of adult cells, including thymocytes, T- and B-cells (in contrast to mammals) and various antigen presenting cells (APC) (Flajnik *et al.* 1990). Nonaka *et al.* (1997b) and Liu *et al.* (2002) found consistent co-segregation across generations of class II genes and class Ia genes in *X. laevis*, supplying strong evidence that these loci are closely linked in a cluster.

X. laevis has a tetraploid genome (4n) consisting of 36 chromosomes with many duplicated gene loci, suggesting that more than two alleles from each locus could be expressed (Du Pasquier *et al.* 1989; Flajnik *et al.* 1999b). However, all but one diploid set of MHC loci have become silenced (Du Pasquier *et al.* 1989; Flajnik *et al.* 1999b) and the MHC remains functionally diploid (Sato *et al.* 1993). Unlike most mammals, which have numerous MHC-class Ia and MHC-class II loci, *Xenopus laevis* have only one MHC-class Ia locus and three MHC-class II loci, regardless of polyploidization (Shum *et al.* 1993; Du Pasquier 2001). The single *Xenopus* MHC-class Ia locus has been found to have alleles belonging to separate lineages as divergent as MHC-class I genes from separate species (humans and mice) (Flajnik *et al.* 1999a) and the PBR appears to be under positive selection with a high ratio of non-synonymous substitutions to synonymous substitutions (*Dn/Ds*) (Flajnik *et al.* 1993). The degree of polymorphism exceeds those found in most vertebrate MHC loci and are comparable to the level of polymorphism found in salmonid fishes (Flajnik *et al.* 1999a; Liu *et al.* 2002; Bos & Waldman 2006).

Most interesting are the changes in expression of MHC-class I and MHC-class II genes at metamorphosis. The immune system of *Xenopus* tadpoles appears to function without the classical MHC-class I molecules expressed on most cell surfaces (Du Pasquier *et al.* 1989; Flajnik *et al.* 1999b). Prior to metamorphosis, only MHC-class II molecules are ubiquitously expressed (Du Pasquier *et al.* 1989), whereas MHC-class Ia transcripts are expressed only in the lung, gill, and intestine, organs with epithelial surfaces in contact with the environment (Salter-Cid *et al.* 1998). After metamorphosis, MHC-class I protein expression increases markedly in other tissues (Salter-Cid *et al.* 1998). Despite their limited MHC-class Ia expression, tadpoles are immunocompetent (Du Pasquier *et al.* 1989), although they are markedly more susceptible to viral infections than are adults (Gantress *et al.* 2003).

In *Xenopus*, unlinked to the cluster of classical MHC loci, non-classical MHC-class Ib genes also exist (Flajnik *et al.* 1993). The *Xenopus* MHC-class Ib loci are not as polymorphic as the MHC-class Ia and class II loci, but are structurally similar to the MHC class Ia (Salter-

Cid *et al.* 1998). MHC-class Ib genes are preferentially expressed in certain tissues, with one family being exclusively expressed in epithelia (Salter-Cid *et al.* 1998). Also analogous to class Ia genes, class Ib transcripts were never detected before metamorphosis (Salter-Cid *et al.* 1998). This suggests that MHC-class Ib genes may interact with or enhance class Ia functions.

Development of Xenopus *Olfaction*

In adult *X. laevis*, olfactory epithelium is housed in three distinct nasal cavities: the principal cavity (PC), the middle cavity (MC), and the vomeronasal organ (VNO) (Petti *et al.* 1999). The olfactory epithelia of these cavities have distinct ultrastructural features (Meyer *et al.* 1996; Hansen *et al.* 1998; Oikawa *et al.* 1998), neuronal specifications (Suzuki *et al.* 1999; Higgs & Burd 2001), biochemical staining patterns (Petti *et al.* 1999; Pinelli *et al.* 2004), olfactory receptor expression (Mezler *et al.* 1999; Hagino-Yamagishi *et al.* 2004), and presumed physiological and behavioural functions (Petti *et al.* 1999).

The formation of the adult olfactory epithelia involves embryonic, larval and metamorphic phases (Franco *et al.* 2001), with certain anatomical, physiological and presumed functional changes that occur during metamorphosis (Petti *et al.* 1999). In *X. laevis* tadpoles, the olfactory system consists of two epithelia in the PC and the VNO (Hansen *et al.* 1998). The sensory epithelium in the MC is formed during metamorphosis (Hansen *et al.* 1998). Physiologically, the epithelium of the larval PC resembles that of the MC in adults in that both are exposed to waterborne odourants (Franco *et al.* 2001). During metamorphosis, the PC undergoes changes in ultrastructure, odourant receptor gene expression, and site of innervation of the receptor neurons that transform it from the larval water-sensing nose to the adult air-sensing cavity (Hansen *et al.* 1998; Petti *et al.* 1999; Franco *et al.* 2001; Higgs & Burd 2001)**.** The MC and the VNO are always exposed to waterborne odourants (Hansen *et al.* 1998) and the VNO does not appear to change function, structure, or innervation during metamorphosis (Higgs & Burd 2001)**.**

Even though the anatomy of *X. laevis* tadpoles indicates that they can only detect waterborne odourants, mammalian-like olfactory receptors (class II) thought to be responsible for the perception of volatile airborne odours, are already detected at stage 49, approximately 12 days after fertilization (Mezler *et al.* 1999, 2001). Nonetheless, the expression of olfactory receptors (class I) thought to be responsible for the perception of water-soluble odorants less than 2 days after fertilization at stage 32 (Mezler *et al.* 1999) reflects the principal anatomy of *Xenopus* tadpoles.

In *X. laevis*, vomeronasal 2 receptor (V2R) genes, which are co-expressed with non-classical MHC class Ib molecules in mice (Loconto *et al.* 2003), are expressed predominantly in the VNO. Phylogenetically, the VNO first appeared in amphibians (Bertmar 1981). In *X. laevis*, the VNO begins to differentiate in tadpoles at stage 41 (Nieuwkoop & Faber 1956; Hansen *et al.* 1998)**.** Between larval stages 46 and 50 (Nieuwkoop & Faber 1956), the co-expression of V2R genes and the G-protein Go commences in the sensory epithelium of the VNO (Hagino-Yamagishi *et al.* 2004). After metamorphosis, the V2Rs and Go are also expressed in the posterolateral PC (Hagino-Yamagishi *et al.* 2004).

Xenopus laevis *Behavioural Ecology*

Unfortunately, very little is known about the ecology of *X. laevis* in its native habitat. However, laboratory studies have offered some insight into the schooling behaviour of *X. laevis* tadpoles. *X. laevis* tadpoles at various developmental stages will form aggregates based solely on the visual presence of conspecific tadpoles (Wassersug & Hessler 1971). Also, *X. laevis* tadpoles tend to orient parallel to their nearest neighbours (Wassersug *et al.* 1981). Furthermore, in his honours thesis, Locker (1989) found that *X. laevis* tadpoles preferentially school with siblings over non-siblings and that they also preferentially school with maternal half-siblings over non-siblings.

Xenopus laevis as a Model Organism for the Study MHC-Biased Behaviours

How kin discrimination behaviour changes through ontogeny has not yet been investigated in *X. laevis.* How the MHC may be involved in facilitating kin recognition has not yet been investigated in any anuran species. Nonetheless, *X. laevis* may serve as an excellent model organism to investigate both kin recognition and MHC-biased behaviours.

Xenopus frogs can produce thousands of tadpoles in a single mating. It is therefore relatively easy to obtain the number of individuals needed to study MHC-biased behaviours thoroughly. *X. laevis* is particularly well suited to studying MHC-biased behaviours as it has only four closely linked MHC loci. The high level of polymorphism at these loci in *X. laevis* (Flajnik *et al.* 1999a; Liu *et al.* 2002; Bos & Waldman 2006) suggests that despite the genetic simplicity of the *Xenopus* MHC, a recognition system based on the MHC or closely linked genes should be sufficient for kin discrimination.

The unique simplicity of the *X. laevis* MHC allows us to type the entire MHC region based on a single genetic marker in the polymorphic sequence of the MHC class Ia PBR. However, genes at closely linked loci, such as the two MHC-class II loci, also may determine any trait that correlates with certain genotypes determined at this marker. Despite the fact that the expression of MHC class Ia molecules in tadpoles is limited, its expression on the epithelial surfaces in contact with the environment may be sufficient for the production of MHC-determined odours.

The olfactory epithelia responsible for the perception of waterborne odourants in *X. laevis* are fully developed within two days after fertilization. Not only does *X. laevis* have a fully developed VNO, which is generally associated with the perception of pheromones that elicit specific behavioural responses, early on in ontogeny, but it also has been shown to express vomeronasal olfactory receptors (V2R). This may be of importance because in mice V2Rs are always associated with the expression of non-classical MHC class Ib molecules,

which in *X. laevis* appear to follow MHC class Ia expression and possibly function in tadpoles (Salter-Cid *et al.* 1998).

In a series of tadpole association preference tests described in this thesis I have further investigated the phenomenon of kin recognition in *X. laevis* tadpoles by examining changes in kin discrimination through ontogeny as well as the role of the MHC in facilitating discrimination.

Chapter 4 : Materials and Methods

Subjects

I conducted all experiments in this thesis on tadpoles from laboratory breedings of either wild caught or purpose-bred strains of *Xenopus laevis* frogs from the University of Canterbury *Xenopus* facility. Wild frogs were captured in irrigation dams in Paarl, Stellenbosch, Somerset West, Caledon, and Wellington in the Eastern Cape Province of South Africa by Guy Pluck in 1999. Laboratory strains of inbred frogs with known MHC types were developed over the past 35 years in the laboratories of Louis Du Pasquier at the Basel Institute of Immunology and Martin Flajnik at the University of Maryland. These strains have been used in immunological studies over the past decades and have known sequences for MHC class I and II alleles within defined haplotypes (*f*, *g*, *j*, and *r*; Flajnik *et al.* 1999a).

Adult frogs with MHC genotypes *fr* and *fg* were obtained from Martin Flajnik. Adult frogs with MHC genotypes *rj* and *gj* were obtained from Louis Du Pasquier. By crossing adult *fr* and *fg* frogs and rearing some of the progeny to sexual maturity I also obtained adult frogs of the genotype *rg*. Because of the poor fecundity of the gj frogs in our lab, progeny from *gj* frogs were not used in any of the studies in this thesis.

Maintenance of Xenopus laevis *Frogs*

All frogs were housed in 60 L polyethylene tanks that had a continuous through-flow of filtered and aerated deep aquifer water at 21°C. Each tank held up to 10 frogs. Tanks were cleaned and records of frog health were kept on a weekly basis. Frogs were fed three times a week with pet turtle food pellets and yellow mealworms (*Tenebrio molitor*) larvae.

Breeding procedure

Prior to breeding, frogs were fed additionally with beef kidney slivers 4 and 2 days. On the day of breeding, between 13:00 and 15:00, I isolated and primed females by injection into the dorsal lymph sac with 0.03 mg Luteinizing Hormone – Releasing Hormone (LH-RH; Argent Chemical Laboratories, Redmont, WA, USA) dissolved in 150 µL of sterilized water. I monitored the cloacae of the frogs from 5 to 8 h after priming. Once cloacae displayed swelling and red colouration from increased blood flow, I injected the females with an additional 0.1 mg LH-RH dissolved in 500 µL of sterilized water and placed them into breeding tanks with plastic grates anchored by rocks to allow fertilised eggs to fall through and avoid contact with and damage by the breeding pair. I injected male frogs with 0.03 mg LH-RH dissolved in 150 µL of sterilized water and placed them into the breeding tanks with their respective female breeding partners. Breeding pairs were left to mate in the breeding tanks overnight.

To avoid fouling of the eggs, I removed the frogs and replaced the water in the breeding tanks with clean filtered and aerated deep aquifer water at 21°C the following day. I moved approximately half of the progeny of wild caught breeding pairs to separate tanks such that stimulus tadpoles and test subjects in later association preference tests could be sourced from different rearing tanks. This ensured that test subjects were always unfamiliar with all stimulus animals regardless of kinship. Two to 3 days later, after the larvae started to extend in shape (stages 20-25; Nieuwkoop & Faber 1956), I removed them from the breeding tanks and placed them into tadpole rearing tanks.

Maintenance of* Xenopus laevis *Tadpoles

I reared tadpoles in 40 L tanks (560 × 340 × 230 mm) holding approximately 200 tadpoles each. After two weeks I reduced the density of holding tanks for the progeny of the wild caught parental crosses to approximately 100 tadpoles per 40 L. Approximately 6 days after breeding, I fed the tadpoles *ad libitum* three times a week with a filtered suspension of

ground nettle tea. Two to three weeks after breeding, I isolated tadpoles from inbred strains of frogs into 1 L polypropylene tricorn beakers.

Genotyping of *Xenopus laevis* at the MHC class-I Locus

I typed parental frogs as well as all stimulus and subject tadpoles based on MHC class I-α1 polymorphisms. As the MHC class I and class II genes demonstrate absolute cosegregation, consistent with strong linkage disequilibrium between these loci (Nonaka *et al.* 1997b; Liu *et al.* 2002), my methods indirectly type the animals at the MHC class II as well as any other closely linked loci. I determined the MHC-genotypes of parental frogs, tadpole test subjects and the stimulus animals by the polymerase chain reaction (PCR) using sequence-specific primers (Invitrogen Life Technologies, New Zealand) (SSP-PCR). I used DNA extracted and purified from blood samples or tail tissue as templates in these reactions.

Obtaining Purified DNA from Adult Frogs

I obtained sterile blood samples from adult *X. laevis* frogs by drawing venal blood directly from the dorsalis pedis arch. I made a 2 mm incision in the dorsal side of the foot such that the dorsalis pedis arch was visible under a dissecting microscope at 15× magnification. I drew Pasteur pipettes out over a Bunsen burner flame and snapped them to produce very fine bevelled needle tips. After coating these Pasteur pipettes with heparin solution to limit blood clotting, I used them to draw sterile blood from the dorsalis pedis arch under a dissecting microscope at 10× magnification. I collected approximately 40 µL of blood from each frog and placed each sample into a 1.5 mL Eppendorf vial (Standard Eppendorf micro test tube 3810X, Eppendorf Germany) containing 20 µL of heparin solution, again to inhibit clotting. I immediately washed the blood with 1 mL Phosphate buffer solution (PBS). After mixing by inversion, I centrifuged (Eppendorf Centrifuge 5804, Eppendorf Germany) the diluted blood for 1 min at 5000 G and discarded the supernatant. I resuspended the remaining blood pellet in 1.5 mL of PBS, which I then split into four

aliquots. I increased the volume of each aliquot to 1.5 mL using PBS. As in the first wash, I centrifuged the aliquots for 1 min at 5000 G and the once again discarded supernatants before storing the blood pellets at -80˚C for subsequent DNA extractions. Louis Du Pasquier personally trained me in these methods. Since amphibian erythrocytes are nucleated (Gambino *et al.* 1984), DNA yield from *Xenopus* blood samples is exceptionally high. I extracted DNA from blood samples by boiling for seven minutes in ten times by volume PrepMan Ultra extraction solution (Applied Biosystems, USA) in 1.5 mL Eppendorf vials. I then centrifuged the vials at 16000 G for 3 min. I purified the supernatant containing the extracted DNA by salt/ethanol precipitation (0.1× 3M Sodium Acetate/ 3× Ethanol).

Obtaining Purified DNA from Tadpoles

Two to 3 weeks after hatching, I clipped a small portion of the tail (which regenerates) of each tadpole from which I extracted genomic DNA using 20 μL PrepMan Ultra extraction solution (Applied Biosystems, Foster City, CA, USA) in 0.5 mL Eppendorf vials. I extracted DNA from tail tissue samples by boiling for 9 min followed by centrifuging at 16000 G for three minutes. I purified the supernatant containing the extracted DNA by salt/ethanol precipitation (0.1× 3M Sodium Acetate/ 3× Ethanol). Tadpoles were not used in experiments for at least another week to allow for tail regeneration.

Sequence Specific Priming – Polymerase Chain Reaction

I MHC-typed individuals by PCR using sequence-specific primers (SSP) that anneal to polymorphic sequences within the MHC class I-α1 domain (coding for the peptide-binding region, PBR) for each of the known alleles. In each SSP-PCR reaction, I included primers that amplify DNA from a conserved region of the MHC (class I-α3 domain) to control for any failed PCR reactions that would otherwise be falsely scored as negative (Krausa *et al.* 1993; Bunce & Welsh 1994; Bunce *et al.* 1995; Gilchrist *et al.* 1998). These control primers produce PCR product in all successful PCR reactions such that false negatives that may occur

in PCR can be identified. I designed primers using Primer3 (Rozen & Skaletsky 2000) from known *X. laevis* sequences (Flajnik *et al.* 1999a; GenBank accession numbers: AF185579, -80, -82, -86). The *f*-haplotype-specific primers (for: GTC TCA GAT CGA GCC TTT GG, rev: TTG CAG GTT CAT CTC TAC CAG T) amplify a 106 base pair fragment. The *g*-haplotype-specific primers (for: GTC TCA GAT CGA GCC TTT GG, rev: GCT CTG ATC CCT TGG CAA T) amplify a 178 base pair fragment. The *j*-haplotype-specific primers (for: GTC TCA GAT CGA ACC TTT GG, rev: CCT CTT CTC CTT TCG CTT T) amplify a 178 base pair fragment. The *r*-haplotype-specific primers (for: AGA TAG AGC ATT TGG GCT GC, rev: ATT CAG GTC CTG CTT TGT CC) amplify a 134 base pair fragment. The control primers (for: TCA CCC TCA TGT AAG AAT TTC AGA, rev: GCT CCA CAT GAC AGG CAT AA) amplify a 236 base pair fragment.

Sequences were amplified on 96-well PCR plates (Axygen Scientific, PCR-96-C) in 12.5 µL PCR reactions, each containing 50 ng of template DNA, PCR reaction buffer (63.6 mM KCl, 127.2 mM Tris-HCl (pH 8.3), 1.9 mM $MgCl_2$), 180µM dNTP (100mM, Eppendorf Germany) and 0.2 units Taq polymerase (Roche Diagnostics, Basel, Switzerland). Primer concentrations varied dependant on the haplotype being tested. Each *f*-specific PCR contained 16.5 pmol of each *f*-specific primer and 3.5 pmol of each control primer. Each *g*-specific PCR contained 12.5 pmol of the *f*-forward primer, 20 pmol of the *g*-reverse primer, and 1 pmol of each control primer. Each *j*-specific PCR contained 15 pmol of the *j*-forward primer, 30 pmol of the *j*-reverse primer, and 0.75 pmol of each control primer. Each *r*-specific PCR contained 21.25 pmol of each *r*-specific primer and 2.5 pmol of each control primer.

The conditions for touchdown PCR in an Eppendorf thermocycler (Eppendorf Mastercycler gradient, Eppendorf Germany) are as follows: denaturation for 90 s at 94˚C, followed by 5 cycles of denaturation for 30 s at 94˚C, annealing for 45 s at 70˚C and primer extension for 30 s at 72˚C, followed by 20 cycles of denaturation for 30 s at 94˚C, annealing

for 50 s at 65˚C and primer extension for 45 s at 72˚C, followed by 5 cycles of denaturation for 30 s at 94˚C, annealing for 1 min at 56˚C and primer extension for 2 min at 72˚C.

I electrophoresed PCR products next to known positives and negatives for 40 min at 70 volts in horizontal 2% agarose gels. Gels were visualized by ethidium bromide fluorescence. An example of how the presence of specific MHC alleles in DNA samples were visualized is presented in Fig. 4.1..

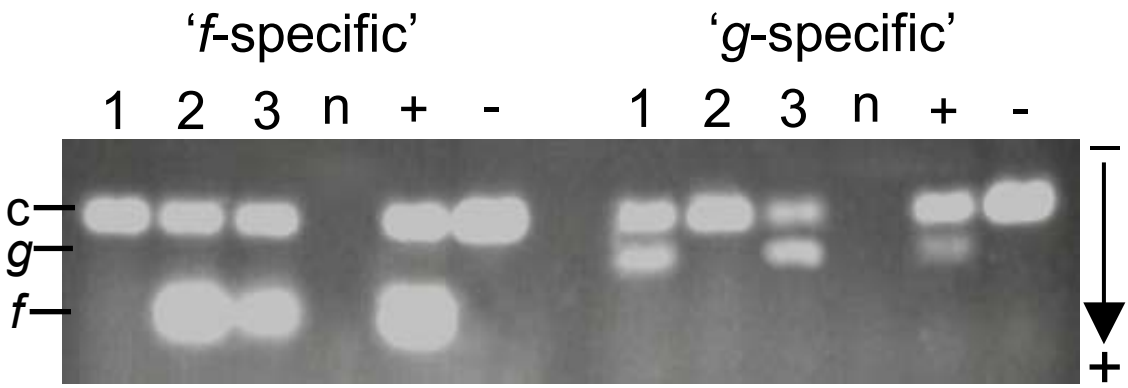

Figure 4.1. Sample of electrophoresed MHC-allele sequence-specific priming (SSP-) PCR products. The MHC genotypes of three tadpoles from an *fg*×*fg* parental cross can be scored from this ethidium bromide agarose gel visualization of allele-specific PCR products (*f* and *g*). The same three samples were tested for both the *f* and *g* MHC class I-α1 domain sequences in two sets of PCR's including control primers (c) from a conserved region of the MHC-class I (α3 domain). Samples were electrophoresed along negative controls (n) and known allele positives (+) and negatives (-). Sample '1' is a *gg* homozygote as only the g-allele sequence amplified. Sample '2' is an *ff* homozygote as only the *f*-allele sequence amplified. Sample '3' is an *fg* heterozygote as both *f* and *g* MHC allele sequences amplified. The same assay was used to score progeny from parents bearing the *r* or *j* alleles using *r*- and *j*-specific primers.

Tadpole Association Preference Tests

I conducted tadpole association choice tests in polypropylene tanks (210 × 140 × 45 mm, Sistema Plastics, Auckland, New Zealand), with removable grey PVC-coated fibreglass (0.028 cm diameter) mesh (7.1 × 5.5 threads/cm) nets (43 × 140 × 45 mm) at each end (Fig 4.2), filled with 1.2 l of filtered deep-aquifer water at 21 ± 1°C. The sides between the mesh

nets were lined with black PVC-coated fibreglass (0.028 cm diameter) mesh, secured with aquarium silicone sealant (Clear Silaflex RTV, Forsoc Limited, Petone, New Zealand), to make the four sides of the test arena between the mesh nets more uniform in texture. This causes the test subjects to spend more time swimming adjacent to the mesh nets rather than swimming mostly back and fourth along the sides between the mesh nets. I placed 10 size-matched stimulus tadpoles in each of the mesh nets. A line drawn in the centre of the test tanks was used to demarcate the two halves of the test arena (124 × 140 × 45 mm). Lighting was diffuse, achieved by reflecting two 100 W incandescent lamps off the ceiling of the test room.

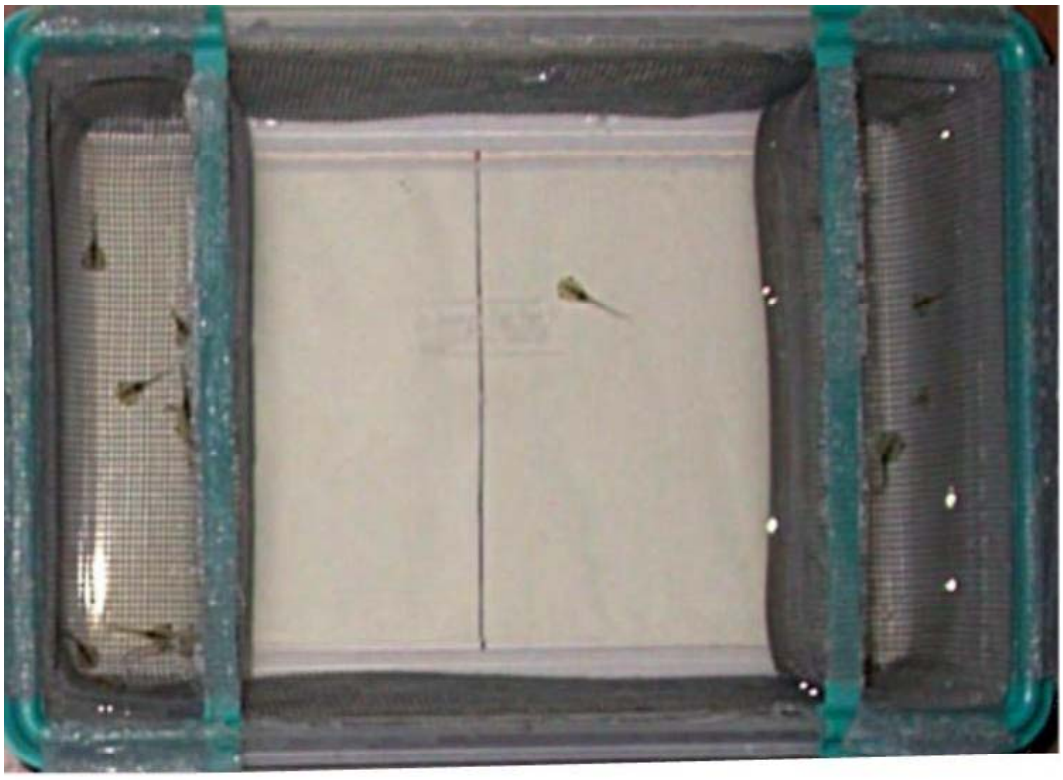

Figure 4.2. Two-way choice tank to test tadpole association preferences

I introduced test subjects by perforated spoon (to limit water volume) into the centre of the apparatus. I allowed tadpoles to acclimate for 5 min, and then tested them for 40 min. I tested each subject twice, reversing the stimulus groups to eliminate any side bias.

Consequently, each tadpole was tested for a total of 80 min (4800 s). I recorded tadpole association tests onto a time-lapse (1/5 speed) VHS recorder (Panasonic AG-TL350), using a CCTV camera (Panasonic WV-BP330/G) with a vari-focal lens (Panasonic WV-LZF61/2) positioned 1 m above the testing apparatus. For each choice test, I aimed at testing forty subjects from each group, however due to limits in available tadpoles, sample sizes vary.

Data Compilation and Analyses

I tracked the movements of subject tadpoles from videotape using EthoVision 3.0 (Noldus Information Technology, Wageningen, Netherlands). I monitored all tracks to remove tracking mistakes (obvious outliers from subject movement trajectories). To ensure that the data were compiled blindly, records of the test subjects and stimulus groups were kept separate until after the tracks were monitored. After combining the tracks of the two choice tests for each subject, I recorded the times spent on either side of a centre line between the stimulus groups for each subject as well as the number of times the centre line was crossed as an indication of movement. An a priori decision was made to remove subjects from analyses that were relatively immobile (below the normal distribution in movements between stimulus groups) and thus had limited interaction with both stimulus groups in the apparatus. Subjects that were quantile outliers in a log transformation of the overall subject movement data (number of times subjects crossed the centre line during the total test period) were omitted from all subsequent analyses. Log transformations of the movement data were conducted to adjust for the positive skew towards high count data that results from the fact that the count data are directional (begins at zero and cannot go below zero). Statistica Box plots, quantile-quantile plots and histograms of the overall data set before transformations, after transformation, and after removal of outliers can be found in Appendix 2. Subjects association preference analyses depended on the study and are given within the methods sections of the data chapters (5-8).

Development of the Behavioural Apparatus

The decision to use the test design described above throughout this thesis was based on a series of trials using different tank designs. I trialed different designs using spare undergraduate laboratory tadpoles from two families. Subjects from only one of the families were used as subjects in these kin discrimination pilot studies. For the apparatus I continued to use, there was a significant schooling bias for sibling tadpoles (n=18, two-tailed paired t-test: $p=0.032$, binomial distribution: $p=0.0481$) in the pilot tests. However, these sibling-biased schooling preferences do not provide evidence for kin discrimination behaviour. They could alternatively have resulted more indirectly from any trait that caused tadpoles to favour the vicinity of the preferred kinship, regardless of the kinship of the test subjects. To determine whether schooling biases result from kin discrimination, subjects taken from both stimulus sibships must be compared. Only if the kin-biased schooling preferences are reciprocal between the kinships, can kin discrimination be inferred. Nonetheless, tadpoles did discriminate between different stimulus groups in the pilot tests which suggests that the apparatus was appropriate for studying schooling biases.

Chapter 5 : Kin Recognition in African Clawed Frog (*Xenopus laevis*) Tadpoles: Ontogeny and Discrimination Cues

Kin-recognition abilities, first demonstrated 25 years ago in toad tadpoles, now appear to be widespread among amphibians. As in other anuran species, I found that African clawed-frog (*Xenopus laevis*) tadpoles preferentially associate with unfamiliar siblings over unfamiliar non-siblings but that this preference varies through ontogeny. The kin-association preference occurs in response to both waterborne and visual cues from stimulus tadpoles. Tadpoles showed no kin-association preference if either waterborne or visual cues of their siblings were occluded. In contrast, pro-metamorphic tadpoles, approaching metamorphosis, demonstrated a reversal in their preference; they preferentially school with non-kin rather than kin. The ontogenetic switch in larval schooling preferences coincides with the onset of thyroid hormone (TH) controlled development and may be indicative of decreased fitness benefits associated with schooling with kin at later developmental stages. These may result from an increase in intraspecific competition, predation, or disease susceptibilities of prometamorphic individuals. Alternatively, the kin avoidance behaviours observed at later larval stages might reflect disassociative behaviour that facilitates inbreeding avoidance at reproductive maturity. This is the first study to find a shift from an association preference for kin to non-kin during amphibian larval development.

INTRODUCTION

Kin discrimination observed in anuran species is commonly interpreted in terms of kin selection (Waldman 2005). Kin selection theory posits that interactions directed towards or away from kin will be selected for if these increase an individual's inclusive fitness (Hamilton 1964). The propensity to preferentially school with kin may increase the inclusive fitness of tadpoles when the benefits of schooling in groups (e.g., cooperative foraging) are disproportionately shared with kin or when the costs of group living (e.g., predation, intraspecific competition) are inequitably distributed among members of a group (Jasieñski 1988; Pakkasmaa & Aikio 2003). The inhibition of growth by crowding in *X. laevis* tadpoles is greater within mixed-sibship groups than within pure-sibship groups (Barribeau 2007). Thus, *X. laevis* tadpoles are likely to benefit from sibling association preferences, as they appear to cope better with environmental stressors, such as crowding, when associating with siblings.

The ability to discriminate kin from non-kin in the absence of prior familiarity has been demonstrated in many vertebrates (Hepper 2005). In anuran amphibians, waterborne cues alone are sufficient for kin discrimination (Blaustein & O'Hara 1982a; Waldman 1985; Gramapurohit *et al.* 2006). Kin discrimination in *X. laevis* tadpoles has been reported (Blaustein & Waldman 1992; Waldman 2005). Moreover, *X. laevis* can form aggregations based on visual cues only (Wassersug & Hessler 1971) or in complete darkness, probably by responding to vibrational cues using their lateral line organs (Katz *et al.* 1981). However, the role of waterborne cues versus visual cues on kin discrimination in *X. laevis* tadpoles has not yet been investigated.

Kin preferences have been found to increase (*Rana sylvatica*, Rautio *et al.* 1991), diminish (*Rana sylvatica*, Waldman 1989; *Rana aurora*, Blaustein *et al.* 1993; *Bufo scaber*, Gramapurohit *et al.* 2006), or persist (*Rana cascadae*, Blaustein *et al.* 1984; *Rana sylvatica*, Cornell *et al.* 1989; *Bufo melanostictus*, Saidapur & Girish 2000) during the late stages of anuran larval development or after metamorphosis. Ontogenetic changes in kin

discrimination may depend on the diverse ecological conditions under which different species breed (Gramapurohit *et al.* 2006) and may correlate with physiological changes that occur as larvae approach metamorphosis.

X. laevis larval development changes drastically beginning at stage 54 (characterized by the differentiation of toes, Nieuwkoop & Faber 1956) at which point further development is controlled by thyroid hormone (TH) (Dodd & Dodd 1976; White & Nicoll 1981; Robertson & Kelley 1996; Furlow & Neff 2006). During the premetamorphic larval stages (37-53; Nieuwkoop & Faber 1956; Cohen & Kelley 1996), development proceeds in the absence of the thyroid gland (Dodd & Dodd 1976; White & Nicoll 1981; Just & Kraus-Just 1996). During the prometamorphic stages (54-62; Nieuwkoop & Faber 1956; (Cohen & Kelley 1996) of larval development, TH induces the remodelling of tadpole tissues into adult tissues (Dodd & Dodd 1976; White & Nicoll 1981). Among other developmental influences, TH initiates hind-limb development (Brown *et al.* 2005) and gonadal differentiation (Iwasawa & Yamaguchi 1984; Kelley 1996), and it seems to be required for the upregulation of major histocompatibility complex (MHC) class I gene expression (Rollins-Smith *et al.* 1997) and changes the structure of the olfactory system (Reiss & Burd 1997). Changes in the olfactory system include the formation of new regions of the olfactory epithelium and olfactory bulb, and a change in olfactory projection patterns (Reiss & Burd 1997). How kin discrimination changes through these developmental stages is of interest because of potential correlations with changes in physiological and anatomical pathways. These correlations may provide insight into the physiological mechanisms that underlie kin recognition.

In this chapter I investigate preferences of *X. leavis* tadpoles to associate with siblings as a function of their developmental stage and of visual and waterborne cues. I do this by examining whether *X. laevis* preferentially associate with siblings or non-sibling at pre-, pro- and post-metamorphic developmental stages and at pre-metamorphic developmental stages with the selective occlusion of waterborne or visual cues.

METHODS

Subjects

Tadpoles were obtained by breeding two pairs of frogs collected in the wild (see chapter 4). Within a day of hatching, approximately half of the progeny from each sibship was transferred to separate 40-litre tanks such that stimulus tadpoles and test subjects in association preference tests could be sourced from different rearing tanks. This arrangement meant test subjects were always unfamiliar with all stimulus animals regardless of kinship. I fed tadpoles 3 times a week *ad libitum* with filtered ground nettle.

Discrimination at different developmental stages

To determine whether *X. laevis* tadpoles discriminate kin at pre-, pro-, or post-metamorphic developmental stages, I conducted eighty-minute choice tests to determine whether individual subjects would associate with either unfamiliar siblings or unfamiliar non-siblings. At premetamorphic (no TH-control) developmental stages (45-53, Niewkoop & Faber 1956; before hind limb toe development), I tested 38 subjects from each of the two sibships. At prometamorphic larval developmental stages (54-59), I tested 15 subjects from sibship 1 and 18 subjects from sibship 2. At postmetamorphic froglet developmental stages (from stage 66), I tested 5 subjects from each of the two sibships. Each subject was tested only once, so at later developmental stages sample sizes were reduced due to a lack of available subjects from the same clutches. Association choice tests commenced two weeks after hatching and were conducted over two months as subjects of appropriate developmental stages became available.

Discrimination based on only either visual or waterborne cues

I tested whether tadpoles can discriminate kin from non-kin in the absence of either visual or chemical cues. I adapted the choice test apparatus described in chapter 4 for testing

schooling preferences while occluding either visual or waterborne cues from stimulus tadpoles.

To test for association preferences based on chemical or other waterborne cues in the absence of visual cues, I constructed partitions through which water could pass while obstructing visual contact between subjects and stimulus groups. These partitions consisted of two adjacent white Perspex panes with 19 and 18 evenly spaced holes (7.5 mm diameter) drilled into them (Fig. 5.1). The holes in the adjacent panes were staggered to each other, such that tadpoles could not see through the holes in both panes. I placed these partitions adjacent to each of the stimulus nets (43 mm from each end), and affixed the same PVC-coated fibreglass (0.028 cm diameter) mesh (7.1 × 5.5 threads/cm) from which stimulus nets were constructed to the subject arena side of the partition such that the wall texture would be similar to that if there were no special partition. I confirmed that subjects in the test arena were exposed to waterborne cues from stimulus tadpoles by testing colour diffusion within the test tank of red and blue dyes placed into each of the stimulus sides of the partitions. Dyes were visible in the test arena within 5 minutes, but remained more concentrated at the stimulus ends of the test arena for over an hour. Thus, tadpoles in the test apparatus were exposed to non-visual cues that tadpoles might discriminate, such as chemical or vibration cues (Katz *et al.* 1981).

To test for association preferences based on visual cues in the absence of waterborne cues, I separated the stimulus tadpoles from the test subjects using transparent Perspex partitions (2.5 mm) instead of mesh nets. I sealed the edges of the partitions to the test tanks 43 mm from each end with silicone, ensuring a watertight barrier between the test arena (120 × 140 mm) and the stimulus groups (43 × 140 mm), such that subjects were only exposed to visual cues from stimulus tadpoles.

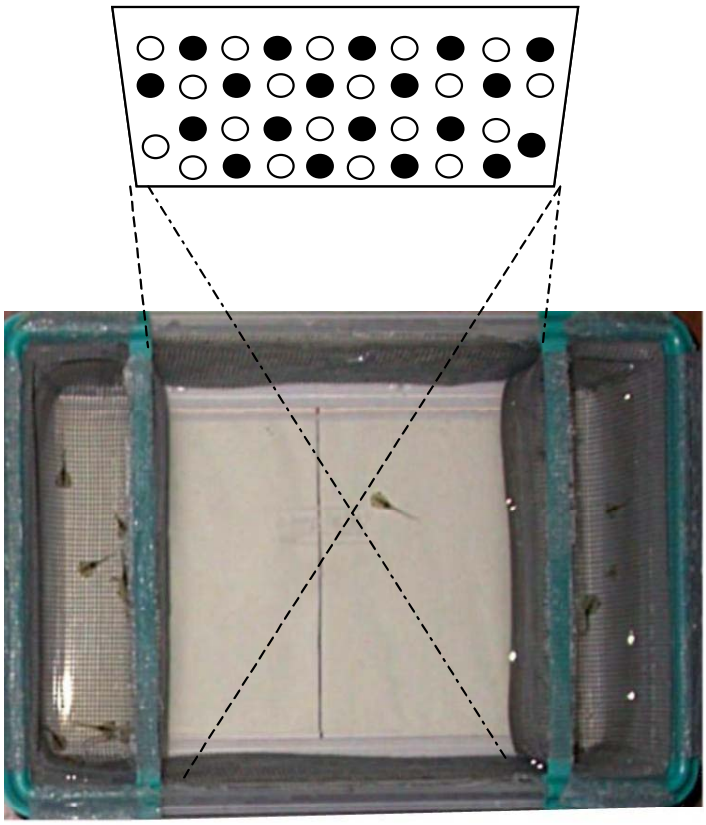

Figure 5.1. Diagram of partitions occluding visual interactions between subject and stimulus tadpoles. Partitions consist of two adjacent panes. Black and white dots represent holes drilled into each of these panes. These double pane partitions were placed between each of the stimulus groups and the subject arena. The subject arena side of each partition was covered with mesh to mimic the texture of the baseline test apparatus

I tested premetamorphic (45-53, Niewkoop & Faber 1956) subjects from the same two sibships used in the ontogenetic study. Thirty-six subjects from sibship 1 and 31 subjects from sibship 2 were tested for association preferences based on only chemical cues. Thirty-five subjects from each of the two sibships were tested for association preferences based on only visual cues.

Data Analysis

For each family, I compared the times that subjects spent near siblings to the times they spent near non-siblings by paired t tests. I tested the the overall effect of kinship on tadpole

association preferences by nested analyses of variance using type III sum of squares (Hill & Lewicki 2006). I compared alternate subjects for the time spent associating with siblings and that spent associating with non-siblings. The effects of kinship and family nested within kinship were included in the analyses of variance. I also compared the proportion of subjects from one of the sibships that spent more time near siblings to the proportion of subjects from the other sibship that spent more time near non-siblings by binomial logistic regression using the likelihood ratio test (The LOGISTIC Procedure; SAS Institute 1995) (Trexler & Travis 1993). All data were initially tested to ensure that they satisfied assumptions of normality. Statistical inferences were based on two-tailed distributions. Unless otherwise indicated, analyses were computed using Statistica 7.1 (Statsoft, Tulsa, OK, USA).

RESULTS

Discrimination at different developmental stages

Premetamorphic tadpoles spent more time associating with unfamiliar siblings than with unfamiliar non-siblings, however this preference only reached significance in one of the two sibships (Sibship 1: $t_{37} = 2.35$, P = 0.024; Sibship 2: $t_{37} = 1.01$, P = 0.32; Fig. 5.2).

Table 5.1. Analysis of variance - at different developmental stages

Source of variation	df	Mean squares	*F* Value	*P*
a. premetamorphic tadpoles				
Kinship*	1	3.88×10^6	4.953	0.029
Sibship(kinship)	2	2.18×10^5	0.278	0.758
Residual error	72	7.84×10^5	—	—
b. prometamorphic tadpoles				
Kinship*	1	7.02×10^6	5.549	0.025
Sibship(kinship)	2	1.02×10^5	0.081	0.923
Residual error	29	1.27×10^6	—	—
c. postmetamorphic tadpoles				
Kinship*	1	1.08×10^6	0.285	0.612
Sibship(kinship)	2	1.03×10^6	0.271	0.771
Residual error	6	3.78×10^6	—	—

*siblings vs. non-siblings

Nonetheless, the overall effect of kinship on schooling preference was significant ($F_{1,72}$ = 4.95; P = 0.029; Table 5.1), whereas variation in kinship-based preferences between sibships was not significant ($F_{2,72}$ = 0.28; P = 0.76; Table 5.1). Most subjects preferred sibling groups (χ^2_1 = 4.48, P = 0.036; Table 5.2).

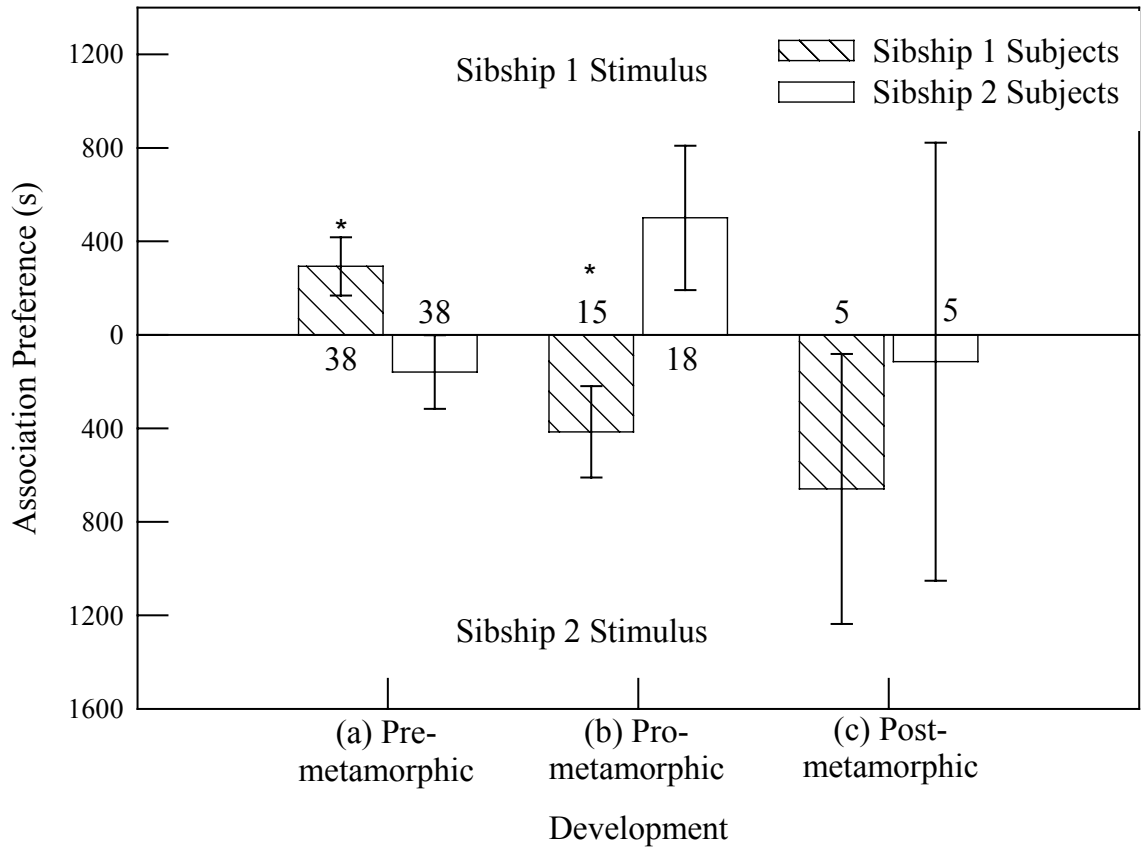

Figure 5.2: (a) At premetamorphic developmental stages (45-53), tadpoles from sibships 1 and 2 demonstrated a significant association preference for their siblings. (b) At prometamorphic developmental stages (54-59), tadpoles from sibships 1 and 2 demonstrated a significant association preference for non-siblings. (c) At postmetamorphic developmental stages (from stage 66), tadpoles from sibships 1 and 2 demonstrated no differential preference for either siblings or non-siblings. Bars represent mean (± SE) differences in preferences between stimulus from sibships 1 and 2. Sample sizes are indicated along the abscissa. *P<0.05 for t tests (two-tailed).

These results demonstrate that *X. laevis* tadpoles preferentially associate with siblings during premetamorphic development. Kin recognition can only be inferred if the kin-biased

schooling preferences are reciprocal between the sibships. Otherwise, association preferences may have resulted from any trait that caused a particular sibship to be preferred, regardless of genetic relatedness to the subject. I demonstrated that the kin-association preferences are reciprocal between the sibships, which implies that subjects recognized their kin. The fact that this experiment was conducted on just two sibships may limit generalisability. Nonetheless, offspring from two independent pairs of wild caught frogs reciprocally preferred their own siblings over the other sibship.

In contrast, prometamorphic tadpoles (stages 54-59) spent more time associating with unfamiliar non-siblings than with unfamiliar siblings, however this preference only reached significance in one of the two sibships (Sibship 1: $t_{14} = 2.13$, P = 0.05; Sibship 2: $t_{17} = 1.62$, P = 0.12; Fig. 5.2). Nonetheless, the overall effect of kinship on schooling preference was significant ($F_{1, 29} = 5.55$; $P = 0.025$; Table 5.1), whereas variation in kinship-based preferences between sibships was not significant ($F_{2, 29} = 0.08$; $P = 0.92$; Table 5.1). Similarly, most prometamorphic subjects spent more time near non-siblings (Table 5.2), although this trend was not significant ($\chi^2_1 = 2.57$, P = 0.11).

Table 5.2. Bivariate stimulus association preferences of subjects at different developmental stages.

	Subject Sibship	Number spending most time near: Sibship 1	Sibship 2	χ_1^2	*P*
Pre-metamorphic	1	26	12	4.38	0.036
	2	17	21		
Pro-metamorphic	1	5	10	2.57	0.11
	2	11	7		
Post-metamorphic	1	2	3	0.40	0.53
	2	3	2		

χ^2 is given for the log likelihood ratio from a binomial logistic regression.

However, recently metamorphosed froglets (from stage 66; postmetamorphic) did not spend more time associating with siblings or non-siblings (Sibship 1: $t_4 = 1.14$, P = 0.31; Sibship 2: $t_4 = 012$, P = 0.91; Fig. 5.2), although this may be due to a small sample size. I found no overall effect of kinship on schooling preference when pooling the data in an analysis of variance either ($F_{1,6} = 0.29$; $P = 0.61$; Table 5.1). Postmetamorphic subjects did not differ in their preferences for siblings and non-siblings (Table 5.2; $\chi^2_1 = 0.40$, P = 0.53).

Discrimination based on only either visual or waterborne cues

Tadpoles spent more time near siblings than non-siblings when presented with waterborne but not visual cues (Fig. 5.3). However, association times did not differ significantly in either sibship (Sibship 1: $t_{36} = 0.26$, P = 0.80; Sibship 2: $t_{30} = 0.77$, P = 0.45). I found no overall effect of kinship on schooling preference when pooling the data in an analysis of variance either ($F_{1,64} = 0.086$; $P = 0.77$; Table 5.3). Similarly, most subjects presented with waterborne cues spent more time near siblings (Table 5.4), although this trend was not significant ($\chi^2_1 = 0.79$, $P = 0.38$).

Table 5.3. Analysis of variance - by cues available to subjects

Source of variation	df	Mean squares	*F* Value	*P*
a. Waterborne & visual cues (same as premetamorphic tadpoles)				
Kinship*	1	3.88×10^6	4.953	0.029
Sibship(kinship)	2	2.18×10^5	0.278	0.758
Residual error	72	7.84×10^5	—	—
b. Waterborne cues				
Kinship*	1	1.69×10^5	0.086	0.770
Sibship(kinship)	2	5.13×10^5	0.262	0.770
Residual error	64	1.96×10^6	—	—
c. Visual cues				
Kinship*	1	3.11×10^6	3.098	0.083
Sibship(kinship)	2	2.57×10^6	2.565	0.085
Residual error	66	1.00×10^6	—	—

*siblings vs. non-siblings

Tadpoles presented with visual but not chemical cues spent more time near non-siblings than near siblings (Fig. 5.3), yet also failed to show a significant preference in either sibhip (Sibship 1: $t_{34} = 0.56$, P = 0.58; Sibship 2: $t_{34} = 1.61$, P = 0.12). I found no overall effect of kinship on schooling preference when pooling the data in an analysis of variance either ($F_{1,66} = 3.10$; $P = 0.083$; Table 5.3). Similarly, most subjects presented with visual cues spent more time near non-siblings (Table 5.4), although this trend was not significant ($\chi^2_1 = 0.79$, $P = 0.38$).

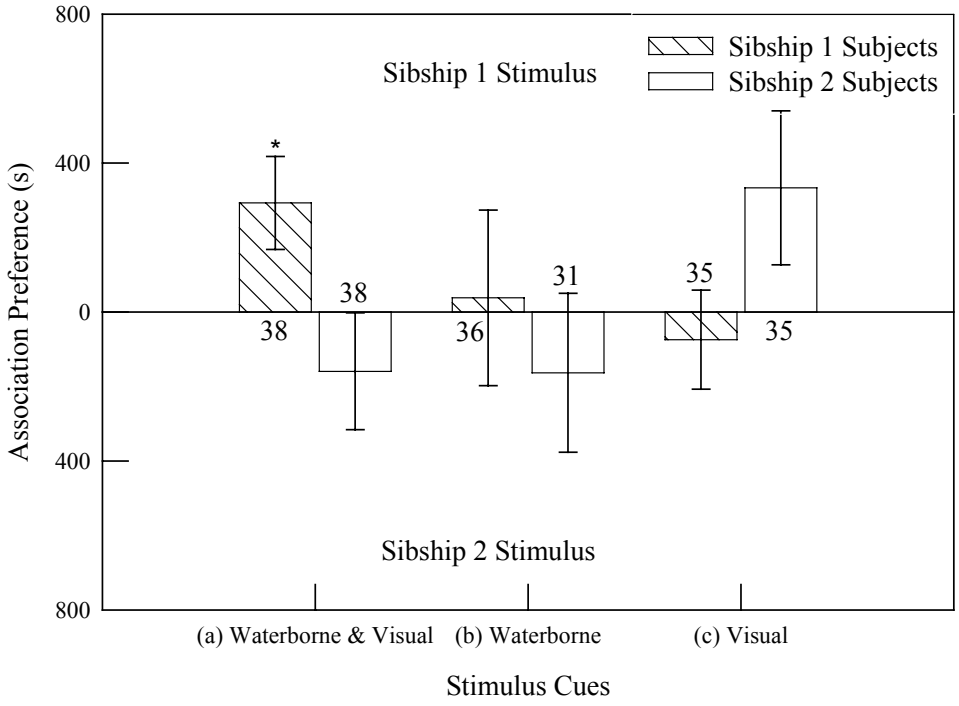

Figure 5.3: Bars represent mean (± SE) differences in preferences between stimulus from sibships 1 and 2. Sample sizes are indicated along the abscissa. *P<0.05 for t tests (two-tailed). (a) For reference, previously presented significant sibling association preferences of premetamorphic tadpoles from sibships 1 and 2 based on both waterborne and visual cues are presented. (b) When subjects were presented with waterborne stimulus cues while occluding visual cues, premetamorphic tadpoles from sibships 1 and 2 demonstrated no association preference for either sibling or non-sibling stimulus tadpoles. (c) When subjects were presented with visual stimulus while occluding chemical cues, premetamorphic tadpoles from sibships 1 and 2 demonstrated a non-significant tendency to associate with non-sibling stimulus tadpoles.

Table 5.4. Bivariate stimulus association preferences of premetamorphic subjects with selective blockade of visual or chemical cues.

	Subject Sibship	Number spending most time near: Sibship 1	Number spending most time near: Sibship 2	χ_1^2	*P*
Waterborne cues	1	19	17	0.79	0.38
	2	13	18		
Visual cues	1	16	19	2.08	0.15
	2	22	13		

χ^2 is given for the log likelihood ratio from a binomial logistic regression.

DISCUSSION

I found a significant kin-association preference in *X. laevis* tadpoles at developmental stages before the differentiation of toes. The kin-association preference apparently requires both visual and waterborne cues from conspecifics as no association preference was found if either was absent. At subsequent larval developmental stages, individuals preferred to associate with non-kin. The small sample size of post-metamorphic subjects limits the ability to determine whether or not this preference persists after metamorphosis. Nonetheless, this is the first study to yield a reversal in kin-biased schooling preferences within amphibian larval development.

Since kin association in my experimental conditions occurs only in response to visual and non-visual, waterborne cues together, there may be both visual and waterborne cues that operate synergistically to direct kin association. Waterborne or visual kinship labels may elicit a behavioural discrimination only when other cues (waterborne or visual) that direct schooling behaviour are also present. *X. laevis* tadpoles form aggregations based solely on the visual presence of conspecific tadpoles (Wassersug & Hessler 1971). Thus aggregation behaviour may require visual cues in *X. laevis*, while kin discrimination may require

waterborne cues. Indeed, waterborne cues alone are sufficient for kin discrimination in anuran amphibians (Blaustein & O'Hara 1982a; Waldman 1985; Gramapurohit *et al.* 2006).

These results demonstrate that the *X. laevis* association preference for siblings at earlier developmental stages switches to an association preference for non-siblings as tadpoles approach metamorphosis. Kin association at earlier developmental stages may facilitate the familiarization with other cues that are reliable predictors of kinship later in ontogeny. A similar functional interpretation has been suggested for kin association tendencies in quail chicks (Waldman & Bateson 1989), which have been found to preferentially outbreed once sexually mature (Bateson 1980, 1982).

The switch from a preference from kin to non-kin coincides with the onset of TH-induced developmental changes. During prometamorphosis, gonads differentiate into ovaries or testes (Iwasawa & Yamaguchi 1984) and secondary sex characteristics begin to develop in response to androgens (larynx; Cohen & Kelley 1996; Robertson & Kelley 1996) and oestrogens (oviducts; Witschi 1971). Furthermore, oestrogen receptor mRNA is first detected during prometamorphosis (Baker & Tata 1990). Hormonal state has been shown to influence, and even reverse odour and association preferences in mice and humans. While female mice demonstrate MHC-disassortative association preferences when in oestrus, female mice in metoestrus preferentially associate with MHC-identical males (Egid & Brown 1989). Similarly, in humans, the estrogenic contraceptive pill seems to reverse preferences for T-shirt odours from MHC-dissimilar individuals (Wedekind *et al.* 1995; Wedekind & Furi 1997).

Although the switch from kin to non-kin preference in the present study was observed well before sexual maturity, it may nonetheless reflect the neuroanatomical and physiological changes that occur between the larval and adult life stages (Furlow & Neff 2006). Kin avoidance in sexually mature frogs is likely to facilitate inbreeding avoidance or optimal outbreeding. In *Bufo americanus*, tadpoles preferentially associate with their siblings

(Waldman 1981) while female toads demonstrate mating preferences for less related males (Waldman 2001). Similarly, whereas juvenile zebrafish (*Danio rerio*) prefer chemical cues of their kin, mature females prefer non-kin (Gerlach & Lysiak 2006). Assortative association preferences also have been found to change to disassortative association preferences based on differences in "fusibility loci" in tunicates. *Botryllus schlosseri* colonies fuse only if they share the same allele at a single fusibility locus (Scofield *et al.* 1982). Larvae settle near colonies that share fusibility alleles (Grosberg & Quinn 1986) whereas fertilization cannot occur between gametes that share these alleles, presumably to prevent inbreeding (Scofield *et al.* 1982).

Ontogenetic changes in kin discrimination of other anurans similarly may be influenced by neuroendocrinological changes. Blaustein *et al.* (1993) found that *Rana aurora* tadpoles cease to discriminate kin at even earlier developmental stages (Gosner stage 28). However, Gramapurohit *et al.* (2006) found that kin-association preferences in *Bufo scaber* tadpoles cease at Gosner stage 37, which, like Nieuwoop and Faber (1956) stage 54 for *Xenopus* tadpoles, is characterized by the differentiation of toes. These studies have suggested that ontogenetic changes in kin discrimination may result from a loss of signal perception or from a greater cost of maintaining the recognition system at later developmental stages (Blaustein *et al.* 1993; Gramapurohit *et al.* 2006). Because of the complex physiological and anatomical differences between pre - and pro-metamorphic tadpoles, I cannot evaluate whether the differences in association preferences resulted from different responses to the same recognition mechanism or from distinct recognition mechanisms (i.e., the loss of one and emergence of another).

Kin avoidance at prometamorphic larval stages may effect dispersal prior to reproductive maturity and thereby decrease the likelihood of inbreeding. Alternatively, the switch from kin association to kin avoidance during *X. laevis* development may reflect changes in fitness associated with the kinship composition of tadpole aggregations. Indeed, wood frog (*Rana sylvatica*) siblings may form either clumped or uniform distributions

depending on the pond, but tadpoles grow faster and larger in ponds in which siblings clump (Halverson *et al.* 2006).

The preferential association with non-kin in prometamorphic *X. laevis* tadpoles might function to direct competition and intraspecific predation (cannibalism) away from kin if these increase at later developmental stages. Kin avoidance may increase an individual's fitness by avoiding competition with (Hamilton & May 1977) or predation on (Pfennig & Collins 1993) kin. Diet may change at later developmental stages, and this might change how tadpoles compete for resources. For example, post-metamorphic individuals that feed on small insects might compete more with one another than filter-feeding tadpoles. Moreover, in *X. laevis*, in which frogs are known to cannibalise tadpoles (Parker *et al.* 1947; Tinsley *et al.* 1996; Measey 1998), kin avoidance may increase metamorphs' inclusive fitness by decreasing the probability that they will cannibalise on closely related individuals.

The preferential association with non-kin at later stages of development may help reduce the spread of disease among more genetically diverse conspecifics (Hamilton 1987; Shykoff & Schmid-Hempel 1991; Tarpy 2003). Tadpoles at later developmental stages might increase both their inclusive and direct fitness by preferentially swarming with non-kin with which they differ in disease susceptibilities. This may be especially important because frogs are naturally immunosuppressed at metamorphosis (Rollins-Smith 1998). Kin association might increase the risk of disease transmission, as host-specific pathogens would be adapted to a similar immune system (Hamilton 1987; Shykoff & Schmid-Hempel 1991; Tarpy 2003). Furthermore, in cannibalistic species, such as *X. laevis*, kin association might lead to the cannibalism of relatives, along with the ingestion of pathogens pre-adapted to a similar immune system (Pfennig *et al.* 1998).

Unfortunately too little is known of the ecology of *X. laevis* to understand how or why intraspecific interactions may change ontogenetically in this species. This highlights the need for further study of *X. laevis* ecology in their native habitat. Further study is also needed to

determine whether *X. laevis* avoid kin in mate choice. Since *X. laevis* is well suited for laboratory research and manipulation, it may prove to be an ideal species for investigating cues involved in amphibian kin recognition.

The testing apparatus that I used to measure kin discrimination in this study can be used to investigate kin discrimination in *X. laevis* tadpoles more closely. In the next chapter, I examine the role the major histocompatibility complex (MHC) on association preferences in *Xenopus laevis* tadpoles at the earlier developmental stages in which they demonstrated a kin preference in this study.

Chapter 6 : MHC-Linked Self-Referent Genotype Matching in *Xenopus laevis* Tadpoles

Major histocompatibility complex (MHC) genes are thought to underpin the ability of vertebrates to recognize their kin. MHC-based discrimination may be favoured by kin selection and fitness benefits that accrue to parents that outbreed including enhanced immunocompetence of MHC-variable offspring. MHC loci exhibit extraordinary polymorphism, so labels that they encode should uniquely identify individuals and serve as markers to map their genetic relationships. However, whether kin recognition is elicited by the MHC, rather than by other genes, has been difficult to ascertain because variation in the MHC correlates closely with overall genetic variation. African clawed-frog (*Xenopus laevis*) tadpoles preferentially school with kin over non-kin, even in the absence of prior social familiarity with them. Here I show that *X. laevis* tadpoles discriminate among familiar full siblings based on differences at the MHC class I locus. Subjects (n=261) from four parental crosses preferred siblings with which they shared MHC haplotypes to those with which they shared no MHC haplotypes. By using only full siblings in experimental tests, I controlled for genetic variation elsewhere in the genome that might influence schooling preferences. As test subjects were equally familiar with all stimulus groups, I conclude that tadpole discrimination involves a self-referent genetic recognition mechanism whereby individuals compare their own MHC type with those of conspecifics.

INTRODUCTION

The ability to distinguish close relatives from non-relatives and more distant kin allows individuals to increase their inclusive fitness even while expressing "altruistic" behaviours that accrue negative direct fitness consequences (Hamilton 1964). Frogs were among the first vertebrates shown to demonstrate kin recognition abilities, most extensively in the context of larval schooling (Waldman 2005) but also in mate choice (Waldman *et al.* 1992; Waldman & Tocher 1998). Kin recognition abilities in amphibians may depend on prior social experience (Waldman 2005) especially during early embryonic development (Hepper & Waldman 1992). Although the recognition of paternal half-siblings in *Rana cascadae* (Blaustein & O'Hara 1982b) and *Rana sylvatica* (Cornell *et al.* 1989) tadpoles implicates genetic influences on recognition labels, the influence of specific genes on larval association preferences has not yet been investigated.

MHC loci are the most polymorphic genes in the vertebrate genome (Piertney & Oliver 2006), which makes them uniquely suited to encode labels of individual and kinship identity. They play a key role in the immune system by producing cellular markers known to facilitate cellular self/non-self recognition (Salter-Cid *et al.* 1998; Gantress *et al.* 2003). They also influence individual odour profiles that facilitate individual 'self/non-self' recognition. Individual MHC type can be discriminated from body odours by rodents (Singh *et al.* 1987; Penn & Potts 1998c), humans (Gilbert *et al.* 1986), and even an electronic nose (Montag *et al.* 2001). Because MHC molecules function as effective 'self-markers' immunologically and uniquely determine individuals' odour profiles, they should be able to serve as effective kin recognition labels.

Indeed, MHC-disassortative mating preferences, which may correlate with kin avoidance preferences, have been observed in Atlantic salmon, sand lizards (*Lacerta agilis*), Savannah sparrows (*Passerculus sandwichensis*), mice and humans (Piertney & Oliver 2006). However, whether such preferences have been selected to immunologically diversify or optimise the resultant MHC-allelic combinations in progeny rather than to facilitate kin

recognition and inbreeding avoidance *per se* remains unclear. Nonetheless, MHC-type discrimination does appear to facilitate nepotistic female choice of communal nesting partners (Manning *et al.* 1992) and parent-progeny recognition (Yamazaki *et al.* 2000) in mice. Furthermore, a preference for siblings sharing MHC alleles has been reported in juvenile Arctic charr (*Salvelinus alpinus*) (Olsen *et al.* 1998; Olsen *et al.* 2002), Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) (Rajakaruna *et al.* 2006) within single sibships.

X. laevis differs from all other vertebrates examined to date in that it has only four closely linked MHC loci (one MHC-class I locus and three MHC-class II loci) (Liu *et al.* 2002), representing a primordial organization of MHC genes (Nonaka *et al.* 1997b). Although *X. laevis* are tetraploid, duplicated MHC genes have become silenced to a diploid number (Flajnik *et al.* 1999b). This limiting of MHC gene numbers may however be compensated for by the level of sequence polymorphism observed at the MHC-class I locus, which is much higher in *X. laevis* than typically found in most well-studied vertebrates (Bos & Waldman 2006).

Consequently, *X. laevis* is a model organism for examining differences across all MHC loci within defined haplotypes (*f, g, j,* and *r*) in discrimination studies. Because all *X. laevis* MHC loci are in complete linkage disequilibrium (Nonaka *et al.* 1997b; Liu *et al.* 2002), I was able to type tadpoles based on polymorphisms in the peptide binding region (PBR) of the MHC-class I locus (see chapter 4) and investigate their association preferences based on the entire MHC region. I tested whether *X. laevis* tadpoles discriminate siblings based on genetic similarity in the MHC. Because only full siblings were tested in this experiment, any consistent association preferences observed must be attributable to the MHC.

METHODS

Using the genotyping methods and association preference choice test procedure described in chapter 4 (Methods), I tested MHC homozygote *X. laevis* tadpoles at developmental stages before hind limb development (stage 54; Nieuwkoop & Faber 1956) for their preference to associate with those full siblings with which they shared MHC haplotypes to those homozygous with different MHC haplotypes. To obtain tadpoles for testing, I crossed four pairs of MHC-identical heterozygote frogs (*rj*×*rj*, *rg*×*rg*, *fg*×*fg*, *fr*×*fr*), from partially inbred lines, to produce multiple sibships consisting of mixed homozygotes and heterozygotes (e.g. *rr, rg*, *gg*). I reared tadpoles with their siblings in groups of 200 in 40-litre tanks for 2 to 3 weeks, after which each individual was placed into a 1-litre cup. I determined the MHC haplotypes of all stimulus and subject tadpoles by PCR from tail-tip tissue (see chapter 4, Methods). Subject sample sizes varied between the different genotypes within the different families depending on the available genotyped progeny of appropriate developmental stage. I tested 18 subjects of the *jj* MHC type and 19 subjects of the *rr* MHC type from the *rj*×*rj* parental cross, 31 subjects of the *gg* MHC type and 41 subjects of the *rr* MHC type from the *rg*×*rg* parental cross, 36 subjects of the *ff* MHC type and 41 subjects of the *gg* MHC type from the *fg*×*fg* parental cross, and 35 subjects of the *rr* MHC type and 40 subjects of the *ff* MHC type from the *fr*×*fr* parental cross.

For each parental cross, I compared the time spent by subjects of one of the MHC-homozygous genotypes associating with MHC-identical siblings to the amount of time spent by subjects of the other MHC-homozygous genotype associating with MHC-different MHC-homozygous siblings using two-sample t tests. I tested the overall effect of MHC similarity on tadpole association preferences by hierarchically nested analysis of variance using type III sum of squares (Hill & Lewicki 2006). I compared alternate subjects for the time spent associating with MHC-identical siblings and that spent associating with MHC-different siblings. The effects of MHC similarity, family nested within MHC similarity, and genotype nested within family and MHC similarity, were included in the analysis of variance. I

compared the number of subjects that spent more time on the side of the tank near the MHC-identical stimulus groups to the number of subjects that spent more time on the side of the tank near the MHC-dissimilar stimulus groups using the binomial distribution. All data were initially tested to ensure that they satisfied assumptions of normality. Statistical inferences were based on two-tailed distributions. Analyses were computed using Statistica 7.1 (Statsoft, Tulsa, OK, USA).

RESULTS

Subjects discriminated among siblings based on their MHC haplotypes. They spent more time with siblings with which they shared MHC haplotypes than with siblings which they shared no MHC haplotypes (*rj*×*rj*: $t_{35} = 2.06$, $P = 0.047$; *rg*×*rg*: $t_{70} = 2.31$, $P = 0.024$; *fg*×*fg*: $t_{75} = 2.56$, $P = 0.012$; *fr*×*fr*: $t_{73} = 2.08$, $P = 0.041$; Fig. 6.1). Because the preferences were consistent among all families, I pooled these results. Overall, the effect of MHC similarity on schooling preference was highly significant ($F_{1, 245} = 22.41$, $P < 0.001$; Table 6.2), whereas variation in MHC-assortative preferences among families ($F_{6, 245} = 0.83$, $P = 0.54$; Table 6.2) or between subjects of both MHC-types tested from each family ($F_{8, 245} = 1.13$, $P = 0.34$; Table 6.2) was not significant. This demonstrates that tadpoles associated preferentially with siblings bearing their own MHC haplotypes rather than with those with particular 'attractive' haplotypes. Across all the families, most subjects preferred MHC-identical siblings to MHC-different siblings (*rj*×*rj*: $P = 0.073$; *rg*×*rg*: $P = 0.005$; *fg*×*fg*: $P = 0.031$; *fr*×*fr*: $P = 0.015$; binomial probabilities; Table 6.1).

Table 6.1. Binomial distributions of individual preferences

Sibship	Number of individuals spending more time near		*P*
	MHC-identical siblings	MHC-different siblings	
rj×*rj*	24	13	0.073
rg×*rg*	48	24	0.005
fg×*fg*	48	29	0.031
fr×*fr*	48	27	0.015

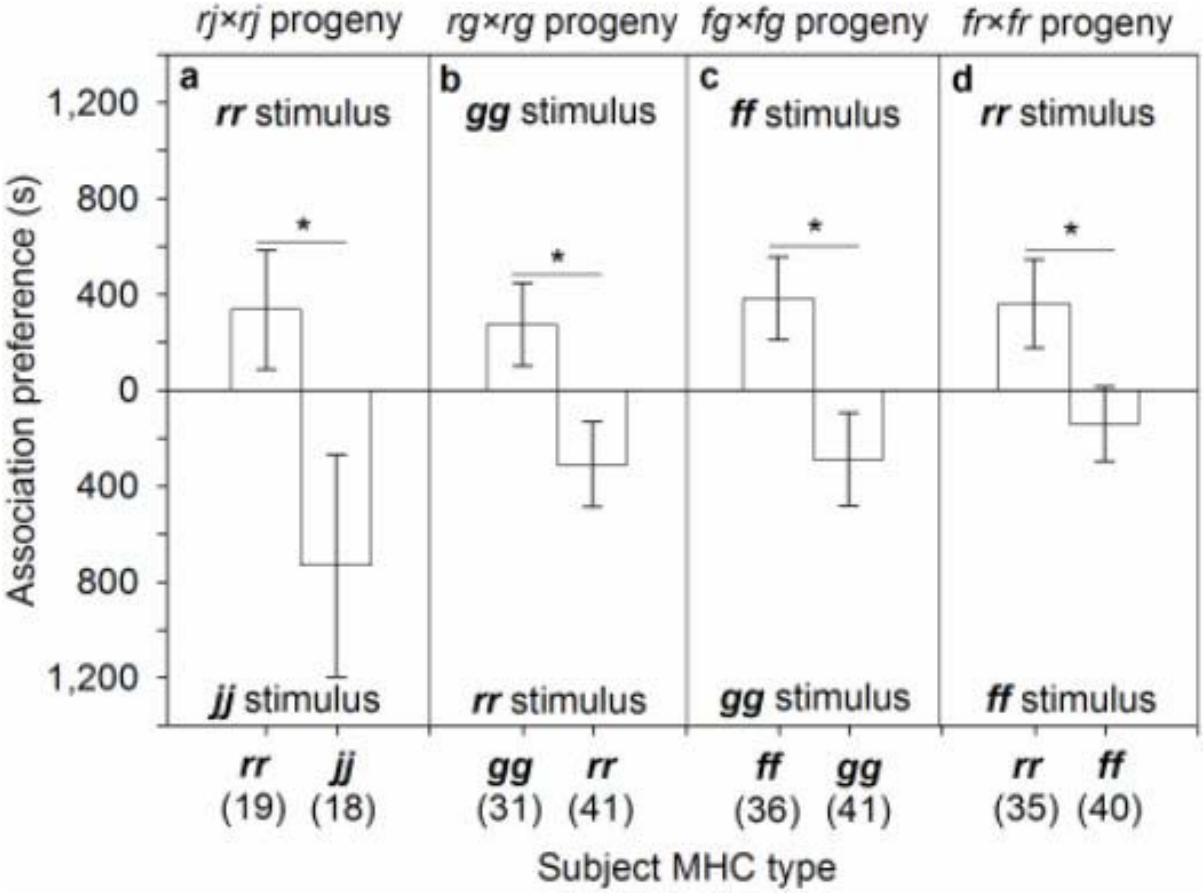

Figure 1. Association preferences (time spent near MHC-identical stimulus group minus time spent near MHC-different stimulus group) of subjects from 4 families (a-d). In all families, MHC-homozygous subjects spent significantly more time associating with MHC-identical siblings than with MHC-different siblings ($P<0.05$; t-tests, two-tailed). Sample sizes indicated in brackets. Means ± s.e.m. are shown.

Table 6.2. Analysis of variance for experimental tests

Source of variation	Degrees of freedom	Mean squares	*F* Value	*P*
MHC similarity	1	7.54×10^{6}	22.406	< 0.001
Family(MHC similarity)	6	2.81×10^{5}	0.834	0.545
Genotype(Family(MHC similarity)	8	3.81×10^{5}	1.131	0.343
Residual error	245	3.37×10^{5}	—	—

DISCUSSION

Tadpoles' association preferences correlated with, and appear to have been determined by, loci within shared MHC haplotypes. In previous studies, MHC-correlated behaviours corresponded to overall genetic similarity (Manning *et al.* 1992; Penn & Potts 1998a) or, conversely, because of limited genetic diversity among subjects, phenotypic differences were restricted to those determined by the MHC (Yamazaki *et al.* 1988, 2000). As I tested only groups of full siblings in this study, I controlled for overall genetic similarity among test subjects and stimulus animals, while maintaining genetic diversity, as expected among siblings, elsewhere in the genome. Hence, the results demonstrate that *X. laevis* tadpoles can discriminate the genotype of the specific MHC class I-α1 domain (coding for the peptide binding region, PBR), other closely linked loci, or both.

The results suggest that tadpoles discriminated MHC similarity by self-referencing. Subjects had interacted freely with their siblings bearing every combination of MHC haplotypes prior to being tested. Previous studies suggest that response biases in anuran larvae are based on templates that incorporate aspects of the early embryonic environment (Waldman 1988, 2005). Tadpoles reared in social isolation from eggs can discriminate between siblings and non-siblings (Blaustein & O'Hara 1981; Waldman 1981, 2005). Furthermore, tadpoles imprint on odorants present in their embryonic environment and subsequently orient towards these odours (Hepper & Waldman 1992). In the current study, however, early social interactions could not have contributed to the formation of recognition templates used for discriminating among disparate MHC types as subjects shared their embryonic and early social environments with siblings bearing either haplotype. The ability of *X. laevis* tadpoles to discriminate among siblings based on MHC-linked differences does not depend on a shared embryonic environment with particular MHC types, but is based on either learning one's own MHC type or an inherent recognition of MHC similarity.

The immune system of *Xenopus* tadpoles functions without MHC-class I molecules expressed on cell surfaces (Salter-Cid *et al.* 1998; Flajnik *et al.* 1999a). This suggests that

loci in linkage disequilibrium with the MHC-class I, such as the MHC-class II, which are expressed on cell surfaces in tadpoles, contribute to the cues involved in directing the observed association preferences. However, MHC-class Ia mRNA transcripts are expressed in tadpoles in the lung, gill, and intestine (Salter-Cid *et al.* 1998). Despite the limited expression of MHC-class Ia in tadpoles, its expression in organs with epithelial surfaces in contact with the environment may be sufficient for the production of MHC-determined odours.

Both MHC class I and class II loci have been shown to influence behavioural discrimination in terrestrial vertebrates and fishes respectively (Piertney & Oliver 2006). MHC-class I molecules bind endogenously derived peptides and class II molecules bind exogenously derived peptides (Bernatchez and Landry 2003). The relative roles of these functionally distinct loci on MHC-type discrimination are not understood. Because the expression of the MHC-class I changes with ontogeny (Salter-Cid *et al.* 1998), *X. laevis* may prove to be a model organism for studying the mechanism by which MHC-type discrimination is achieved – whether through the release of MHC-class I molecules from epithelial tissues in contact with the environment or through the release of volatile aromatics or peptide products associated with either MHC-class I or class II expression.

The MHC discrimination demonstrated by tadpoles is more likely to be kin-selected than an incidental consequence of MHC expression. Unlike MHC-biased mating preferences, which may confer direct fitness benefits by increasing immunocompetence in offspring (Landry *et al.* 2001; Piertney & Oliver 2006), MHC-assortative schooling is more likely to decrease an individual's direct fitness as MHC-similar individuals share disease susceptibilities (Gantress *et al.* 2003). The inclusive fitness benefits associated with kin discrimination thus must outweigh the decreased direct fitness consequences of MHC-assortative schooling.

This study provides robust evidence of social discrimination based on MHC similarity without the possibility of confounding environmental and genetic factors. Furthermore, because tadpole association preferences correlate with MHC-class I-α1 domain similarity, even when subjects are equally familiar with all sibling MHC types, this study provides evidence for self-referent matching of MHC determined phenotypes. The tadpoles use highly polymorphic matching loci to socially discriminate among conspecifics, which should permit the effective discrimination of kin by genetic similarity detection (Grafen 1990). In the next chapter I test whether single haplotype differences are sufficient to facilitate MHC-assortative preferences, and investigate whether preferences correlate with amino-acid differences at the peptide binding region (PBR) of the MHC loci.

Chapter 7 : MHC-Type Discrimination in *Xenopus laevis* Tadpoles Correlates with the Number of Amino Acid Differences in the Peptide Binding Region of the MHC class I and class II

African clawed-frog (*Xenopus laevis*) tadpoles preferentially school with kin over non-kin. Kin recognition in fishes, lizards, birds, rodents, and even humans uses highly polymorphic major histocompatibility complex (MHC) genes. In the previous chapter I found that MHC-homozygous subjects preferentially schooled with MHC-identical siblings over those siblings with which subjects shared no MHC haplotypes. In this study I tested tadpoles' association preferences among siblings based on variable numbers (0, 1, or 2) of shared haplotypes. By mating MHC-heterozygous parents, I obtained families of full siblings that shared different numbers of MHC haplotypes. I found a significant overall schooling preference for MHC-identical siblings over siblings with which subjects shared only one MHC haplotype, but inconsistent preferences among individual sibships. The strength of tadpoles' MHC-assortative schooling preferences significantly correlated positively with amino acid differences in the peptide binding region (PBR) of both the MHC class I and II. Since MHC-PBR polymorphisms determine the pool of peptides that can serve as ligands for MHC molecules, these findings support the hypothesis that MHC peptide ligands mediate MHC type discrimination.

INTRODUCTION

In chapter 6, I found that MHC-homozygous premetamorphic *X. laevis* tadpoles preferentially associate with MHC-identical siblings over siblings with which they shared no MHC haplotypes (2 vs. 0). Such discrimination may depend on the number of shared MHC haplotypes. Similarly, the ability of *X. laevis* tadpoles to discriminate among MHC-disparate individuals may correlate with the number of shared amino acids in the MHC-PBR.

Testing tadpole association preferences among stimulus animals that differ by only one haplotype might shed light into whether association preferences are based on haplotype differences or similarities. If subjects demonstrate a preference for stimulus animals with no dissimilar haplotypes over those with one dissimilar haplotype (2 vs. 1 shared haplotypes) but show no preference for stimulus animals with one dissimilar haplotype over those with two dissimilar haplotypes (1 vs. 0 shared haplotypes), discrimination is likely to result from recogniziing dissimilar haplotypes. Conversely, if subjects demonstrate a preference for stimulus animals with one similar haplotype over those with no similar haplotypes (1 vs. 0 shared haplotypes) but show no preference for stimulus animals with two shared haplotypes over those with only one shared haplotype (2 vs. 1 shared haplotypes), discrimination is likely to result from recognizing similar haplotypes.

MHC-based odour discrimination in mice occurs if the different MHC-mutant strains differ by three but not by one or two amino acids in the MHC-peptide binding region (PBR) (Carroll *et al.* 2002). Likewise, Atlantic salmon choose their mates to increase the heterozygosity of their offspring at the MHC and these mating preferences correspond to the genetic distances between the alleles as determined by amino acid differences in the PBR (Landry *et al.* 2001).

In this chapter, I investigate tadpole association preferences among siblings based on the number of shared MHC-haplotypes (0, 1, or 2). I also examine whether the observed

association preferences among siblings correlate with differences in numbers of shared amino acids in the PBR of the MHC-class I and class II.

METHODS

Association preferences based on shared MHC haplotypes

Using the genotyping methods and association preference choice test procedure described in chapter 4 (Methods), and the same four parental lines used in chapter 6, I tested whether (a) MHC-homozygous tadpoles preferentially associate with MHC-identical siblings over siblings with which they shared only one MHC haplotype (2 vs. 1 – MHC-homozygote subjects; $N = 188$), (b) MHC-homozygous tadpoles preferentially associate with siblings with which they shared only one MHC haplotype over siblings with which they shared no MHC haplotypes (1 vs. 0 – MHC-homozygote subjects; $N = 202$), and (c) MHC-heterozygous tadpoles preferentially associate with siblings with which they shared both MHC haplotypes over siblings with which they shared only one MHC haplotype (2 vs. 1 – MHC-heterozygote subjects; $N = 213$). Subject sample sizes for the two replicate test groups from each of four sibships used in each of these choice tests (a-c) are indicated in Table 7.1.

For each choice test type (a-c), I evaluated the effect of MHC similarity on tadpole association preference by hierarchically nested analyses of variance using type III sum of squares (Hill & Lewicki 2006). I compared alternate subjects for the time they spent associating with MHC-similar siblings and the time they spent associating with MHC-dissimilar siblings. For each of the subject choice tests (a-c), the effects of MHC similarity, genotype nested within MHC similarity, and sibship nested within genotype and MHC similarity, on association times were included in the analysis of variance.

To determine whether particular genotypes demonstrated MHC similarity based association preferences, I further partitioned sum of squares by specific genotype using

orthogonal contrasts with Bonferroni adjusted alpha levels of 0.0056 (0.05/9) per contrast (see Table 7.2).

I compared the number of subjects that spent more time on the side of the tank near the MHC-similar stimulus group to the number of subjects that spent more time on the side of the tank near the MHC-dissimilar stimulus group using the binomial distribution with Bonferroni adjusted alpha levels of 0.0021 (0.05/24) per test. All data were initially tested to ensure that they satisfied assumptions of normality. Statistical inferences were based on two-tailed distributions. Analyses were computed using Statistica 7.1 (Statsoft, Tulsa, OK, USA).

Association preferences based on MHC-PBR amino acid similarity

For each choice test ('2 vs. 0', '2 vs. 1', '1 vs. 0', and '2 vs. 1-heterozygous subjects'), in each of the four families, I calculated measures of (i) MHC-class I PBR (α1 and α2 domains) amino acid similarities (Flajnik *et al.* 1999a) between subjects, and (ii) MHC-class II PBR (α1 and α2 domains of the DAA and DBA loci) amino acid similarities (Liu *et al.* 2002) between subjects. I did not include the MHC class II DCA locus in the analysis as it has only been partially sequenced for the *f* haplotype and is expressed in very low amounts, if at all (Liu *et al.* 2002).

For each choice test, I counted the number of shared amino acids in the MHC class I-α1 and α2 domains and the MHC class II *A* α1 and α2 domains of the DAA and DBA loci between the test subject and each of the stimulus groups, and expressed these as a percentage of total number of amino acids in the PBR (see Appendix 2d). I determined the stimulus amino acid differentials by subtracting the percentage amino acid similarity of the more MHC-dissimilar stimulus group from the percentage amino acid similarity of the more MHC-similar stimulus group (Appendix 2e). These stimulus differentials are a function of the type of choice test ('2 vs. 0', '2 vs. 1', '1 vs. 0', '2 vs. 1 - heterozygous subjects') as well as of the haplotype differences within the different parental lines. Since association preferences were

not normally distributed, I used Spearman rank order correlations (Statistica 7.1) to correlate the magnitude of subjects' preference for the more MHC-similar stimulus group with the appropriate amino acid stimulus differentials.

RESULTS

Association preferences based on shared MHC haplotypes

Tadpoles associated preferentially with siblings with which they shared both MHC haplotypes over siblings with which they shared only one MHC haplotype ('2 vs. 1'; $F_{1, 172} = 4.34$, $P = 0.039$; Table 7.2a). However, preferences were inconsistent among the MHC types

Table 7.1. Tadpole association preferences

Parental line	Stimulus MHC-types	Subject MHC-type*	Association preference (s) for MHC-similar stimulus (Mean ± SE)**	Number of individuals		$P^{\dagger}$
				MHC-similar	MHC-dissimilar	
a. Stimulus sharing 2 vs. 1 mhc haplotypes (MHC-homozygote subjects)						
rj × *rj*	*jj* vs. *rj*	*jj* (10)	547.5 ± 266.3	2	8	0.065
rj × *rj*	*rr* vs. *rj*	*rr* (9)	-40.7 ± 518.4	5	4	0.754
rg × *rg*	*rr* vs. *rg*	*rr* (25)	31.3 ± 227.3	11	14	0.557
rg × *rg*	*gg* vs. *rg*	*gg* (31)	-36.5 ± 186.3	13	18	0.377
fg × *fg*	*ff* vs. *fg*	*ff* (23)	37.5 ± 120.8	10	13	0.541
fg × *fg*	*gg* vs. *fg*	*gg* (23)	477.2 ± 192.9	14	9	0.308
fr × *fr*	*ff* vs. *fr*	*ff* (40)	201.3 ± 146.4	20	20	1.000
fr × *fr*	*rr* vs. *fr*	*rr* (27)	-113.1 ± 187.2	11	16	0.345
b. Stimulus sharing 1 vs. 0 mhc haplotypes (MHC-homozygote subjects)						
rj × *rj*	*jj* vs. *rj*	*rr* (8)	405.4 ± 187.4	6	2	0.180
rj × *rj*	*rr* vs. *rj*	*jj* (16)	38.6 ± 221.0	7	9	0.629
rg × *rg*	*rr* vs. *rg*	*gg* (34)	-39.7 ± 147.0	16	18	0.736
rg × *rg*	*gg* vs. *rg*	*rr* (22)	687.5 ± 251.6	15	7	0.093
fg × *fg*	*ff* vs. *fg*	*gg* (30)	-110.1 ± 179.1	13	17	0.473
fg × *fg*	*gg* vs. *fg*	*ff* (20)	-156.1 ± 193.1	8	12	0.383
fr × *fr*	*ff* vs. *fr*	*rr* (32)	119.2 ± 159.7	17	15	0.728
fr × *fr*	*rr* vs. *fr*	*ff* (40)	-27.6 ± 101.7	20	20	1.000
c. Stimulus sharing 2 vs. 1 mhc haplotypes (MHC-heterozygote subjects)						
rj × *rj*	*jj* vs. *rj*	*rj* (22)	-173.6 ± 299.0	10	12	0.678
rj × *rj*	*rr* vs. *rj*	*rj* (28)	-494.3 ± 202.5	8	20	0.024
rg × *rg*	*rr* vs. *rg*	*rg* (26)	50.6 ± 235.3	16	10	0.248
rg × *rg*	*gg* vs. *rg*	*rg* (27)	-432.7 ± 235.0	8	19	0.036
fg × *fg*	*ff* vs. *fg*	*fg* (26)	-364.6 ± 225.6	12	14	0.701
fg × *fg*	*gg* vs. *fg*	*fg* (25)	2.8 ± 202.1	15	10	0.327
fr × *fr*	*ff* vs. *fr*	*fr* (29)	140.1 ± 226.1	16	13	0.585
fr × *fr*	*rr* vs. *fr*	*fr* (30)	95.5 ± 278.6	14	16	0.720

*Sample sizes (*N*) are indicated in brackets

**Positive 'means' indicate preference for MHC similar and negative 'means' indicate preference for MHC dissimilar

†Binomial test

(Table 7.1a). MHC-similarity based association preferences did not differ significantly between genotypes or between the different sibship groups of the same MHC-homozygous genotypes (Table 7.2a).

Table 7.2. Analysis of variance for experimental tests

Source of variation	df	Mean squares	*F* Value	*P*
a. 2 vs. 1 shared haplotypes (MHC-homozygote subjects)				
MHC similarity	1	1.08×10^{6}	4.341	0.039
Genotype(MHC similarity)	3	1.44×10^{5}	0.580	0.628
Orthogonal contrasts:				
rr - MHC similarity contrast	1	1.15×10^{4}	0.047	0.829
gg - MHC similarity contrast	1	6.43×10^{5}	2.597	0.109
ff - MHC similarity contrast	1	2.15×10^{5}	0.831	0.363
Sibship(Genotype(MHC similarity))	8	1.59×10^{5}	0.644	0.740
Residual error	172	2.47×10^{5}	—	—
b. 1 vs. 0 shared haplotypes (MHC-homozygote subjects)				
MHC similarity	1	3.53×10^{5}	1.631	0.203
Genotype(MHC similarity)	3	2.27×10^{4}	0.114	0.952
Orthogonal contrasts:				
rr - MHC similarity contrast	1	1.82×10^{6}	9.157	0.003
gg - MHC similarity contrast	1	1.97×10^{4}	0.099	0.753
ff - MHC similarity contrast	1	1.12×10^{5}	0.565	0.453
Sibship(Genotype(MHC similarity))	8	1.96×10^{5}	0.988	0.447
Residual error	186	1.99×10^{5}	—	—
c. 2 vs. 1 shared haplotypes (MHC-heterozygote subjects)				
MHC similarity	1	6.56×10^{5}	1.691	0.195
Genotype(MHC similarity)	3	4.35×10^{5}	1.119	0.342
Orthogonal contrasts:				
fr - MHC similarity contrast	1	2.01×10^{6}	0.518	0.472
rg - MHC similarity contrast	1	4.67×10^{5}	1.203	0.274
fg - MHC similarity contrast	1	2.16×10^{4}	0.056	0.814
Sibship(Genotype(MHC similarity))	8	4.78×10^{5}	1.230	0.283
Residual error	197	3.88×10^{5}	—	—

Overall, MHC-homozygous subjects did not associate preferentially with siblings with which they shared only one MHC haplotype over those with which they shared no haplotypes ('1 vs. 0'; $F_{1, 186} = 1.63$, $P = 0.20$; Table 7.2b). However, *rr* tadpoles preferred siblings with which they shared one *r* haplotype over those lacking the *r* haplotype ($F_{1, 186} = 9.16$, $P = 0.003$).

MHC-heterozygous subjects failed to show significant association preferences for siblings with which they shared both MHC haplotypes over those homozygous for only one of the subject's MHC haplotypes ('2 vs. 1 – heterozygous subjects'; $F_{1, 197} = 1.69$, $P = 0.20$; Table 7.2c).

Association preferences based on MHC-PBR amino acid similarity

Association preference times for the MHC-similar stimulus groups correlated positively and significantly with the difference in shared amino acid residues at the MHC-class I PBR (α1 and α2 domains) ($r_s = 0.15$, $t_{862} = 4.51$, $P < 0.001$; Fig 7.1a), Differences in shared amino acid residues at the MHC-class II PBR (α1 and α2 domains of the DAA and DBA loci) also correlated significantly with behavioural preferences for MHC haplotype similarity ($r_s = 0.14$, $t_{862} = 4.30$, $P < 0.001$; Fig. 7.1b). For both the MHC class I and class II, the greater the PBR amino acid stimulus differential, the more time test subjects spent with the MHC-similar stimulus groups, indicating a greater tendency to discriminate between the MHC-disparate stimulus groups.

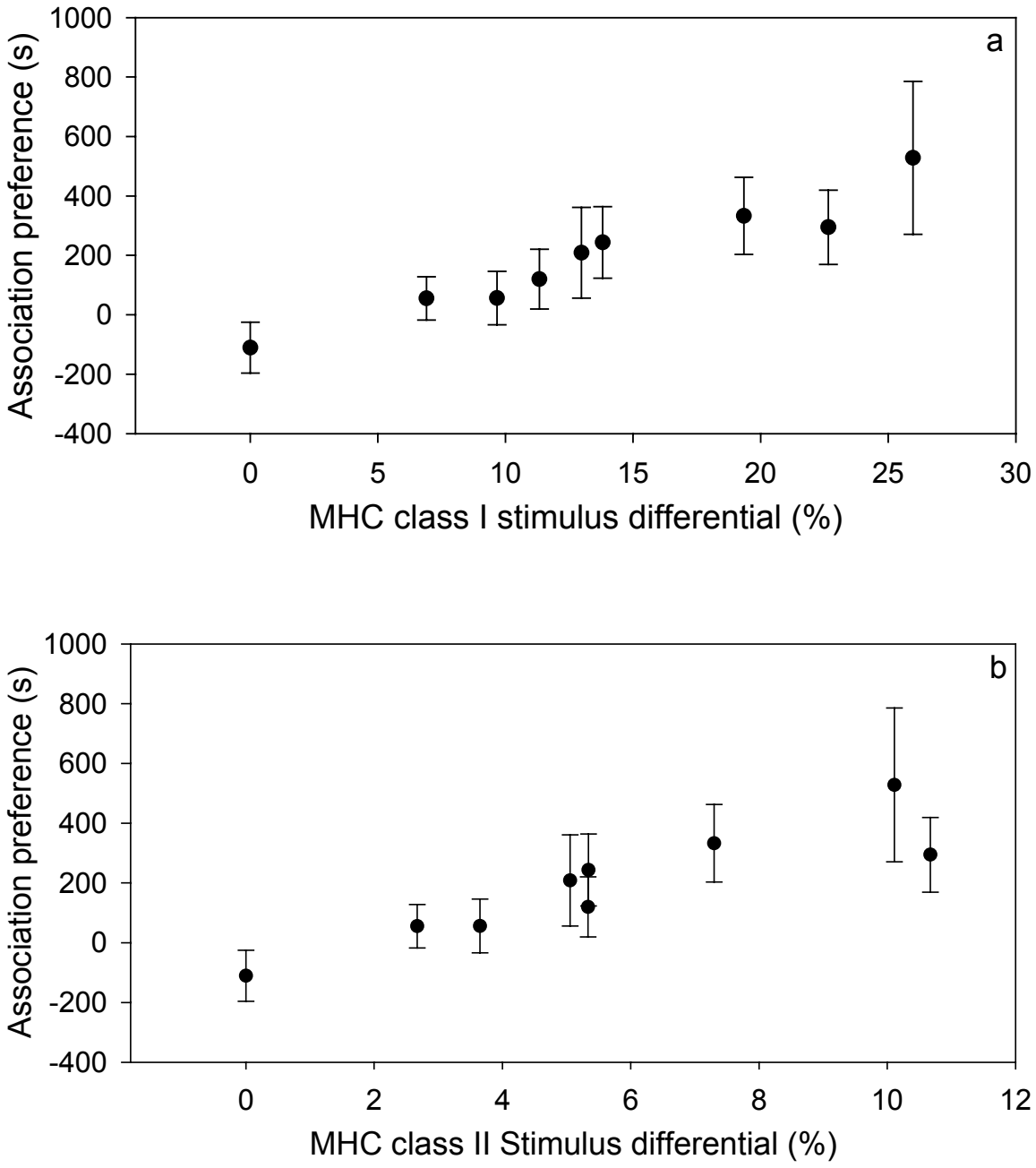

Figure 7.2: Mean association preferences for MHC-similar tadpoles of subjects based on the (a) MHC class Iα PBR stimulus differential of the choice test (r = 0.15, *N* = 864, *P* < 0.001,). (b) MHC-class IIα PBR (DAA and DBA) stimulus differential of the choice test (r_s = 0.14, *N* = 864, *P* < 0.001,). Stimulus differentials are the percentage of shared amino acids of the MHC-similar stimulus group minus the percentage of shared amino of the MHC-dissimilar stimulus group. Error bars denote ± SE.

DISCUSSION

These results demonstrate that the ability of *X. laevis* tadpoles to discriminate between MHC-disparate siblings, as demonstrated in chapter 6, is diminished when given a choice between sibling stimulus groups that both either share MHC haplotypes or bear MHC haplotypes not shared by the test subjects. MHC homozygous tadpoles demonstrated a significant overall association preference for MHC-identical siblings over siblings with which they shared only one MHC haplotype (2 vs. 1 shared haplotypes). However, this preference was not as strong as those found in chapter 6, in which the dissimilar stimulus groups shared no MHC haplotypes (2 vs. 0 shared haplotypes). Among MHC homozygous tadpoles, only *rr* subjects from three different parental crosses demonstrated a significant association preference for siblings with which they shared one MHC haplotype over siblings with which they shared no MHC haplotypes (1 vs. 0 shared haplotypes). MHC heterozygous tadpoles showed no association preference for either MHC-identical heterozygous siblings or siblings homozygous for one of the MHC haplotypes shared with the subjects (2 vs. 1 shared haplotypes – heterozygous subjects).

MHC-type discrimination depends on variable differences in numbers of dissimilar haplotypes rather than in numbers of shared haplotypes. In the two choice tests in which tadpoles demonstrated significant MHC-assortative association preferences (2 vs. 0 and 2 vs. 1), subjects were presented with stimulus groups that either bore or did not bear any MHC-dissimilar haplotypes. In contrast, in the two choice tests in which tadpoles demonstrated no significant MHC-assortative association preferences (‘1 vs. 0’ and ‘2 vs. 1 – heterozygotes’), subjects were presented either with stimulus groups that both bore MHC-dissimilar haplotypes (1 vs. 0) or with stimulus groups that both bore no MHC-dissimilar haplotypes (2 vs. 1 – heterozygotes).

The preference found among tadpoles of only one of the genotypes (*rr*) for siblings sharing one MHC haplotype over those sharing no MHC haplotypes is difficult to interpret, yet raises interesting questions. MHC type discrimination may be enhanced in certain

contexts for certain haplotypes as a function of more general advantages in associating with that particular genotype. For example, if one haplotype conferred a greater resistance to environmental pathogens than the others, its preferential association with other individuals bearing the same haplotype may have been influenced by the greater immunocompetence conferred by the preferred haplotype.

Tadpole preferences for MHC haplotypes correlate with PBR-amino acid differences, which reflect ligand anchor residues that the MHC molecules can bind. Sticklebacks, which have multiple MHC-loci, are most attracted to odours of potential mates with the greatest diversity of MHC-alleles across all loci rather than MHC-dissimilar individuals *per se* (Reusch *et al.* 2001). Milinski *et al.* (2005) also found that these preferences were associated with diversified MHC peptide ligands, which are a consequence of the differences in ligand anchor residues in the PBR of MHC molecules. These anchor residues determine the pool of MHC-bound peptide ligands of nine amino acids (9-mers) typically used in MHC presentation (Rammensee *et al.* 1999; Burroughs *et al.* 2004; Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005).

The MHC-PBR determined pool of peptides that can serve as ligands for MHC molecules (Rammensee *et al.* 1999) may determine individual odour profiles (Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005). Considering the number of distinct 9-mers that are possible in the human proteome, Burroughs *et al.* (2004) calculated that the probability of a foreign peptide being identical to a self-peptide is about 0.2%. These results indicate that these small subunits carry sufficient information for self/non-self discrimination (Burroughs *et al.* 2004). Leinders-Zufall *et al.* (2004) demonstrated that pregnant mice are more likely to undergo pregnancy block if exposed to synthesized 9-mers based on disparate rather than familiar MHC peptide ligands, Similarly, Milinski *et al.* (2005) were able to predictably modify mate choice decisions of female sticklebacks by adding different combinations of the synthetic 9-mer peptides. My finding that amino acid differences in the PBR of both MHC

class I and II correlate with the amount of association preference observed in *X. laevis* tadpoles supports the hypothesis that MHC peptides facilitate MHC type discrimination.

Another recent study provides a possible functional explanation for why *X. laevis* tadpoles preferentially associate with conspecifics based on MHC similarity in the PBR. *X. laevis* tadpoles exhibit optimal development with exposure to water conditioned from frogs (frog holding tank water) with intermediate to high MHC similarity to themselves in the PBR (Barribeau 2007). Thus, the preferential association with conspecifics that share greater similarity in the PBR may result in optimal developmental rates.

The correlation between PBR amino-acid similarity at the MHC-class Iα and class IIα loci and association preference found in this study suggests that these loci may be responsible, at least in part, for the observed association preferences in *X. laevis* tadpoles. If other closely linked loci are responsible for the observed association preferences, the relative polymorphisms at these loci should be similar to those found in the α1 and α2 domains of the MHC class I and the MHC class II DAA and DBA loci.

Since the observed behavioural preferences correlate with amino acid similarities of both the MHC class I and class II, this study provides no further insight into the relative roles of these loci in directing behavioural biases. MHC class I molecules have not been detected at the developmental stages at which *X. laevis* tadpoles were tested in this study. Nonetheless, because MHC class I mRNA transcripts have been detected in organs with epithelial surfaces in contact with the environment, such as lungs, gills, and intestines (Salter-Cid *et al.* 1998), the MHC class I locus is as likely as the MHC class II loci to be involved in MHC-type discrimination. The function of these transcripts in the absence of detectible MHC-class I molecule expression is not clearly understood. However, the presence of adult levels of class Iα mRNA in tadpole gill, an organ that disappears at metamorphosis, suggests that the transcripts expressed in tadpoles are not merely a function of class I protein expression in adults. Since the MHC-class I transcripts in tadpoles are limited mainly to tissues in contact

with the external environment, their protein products may be excreted into the aquatic environment and may be sufficient for the transmission of MHC-specific cues. Further studies on the function of MHC-class I transcripts in tadpoles and on the excreted products of *X. laevis* tadpoles may provide insight into how MHC-specific cues are produced and excreted.

Other factors such as health and genomic differences are likely to be important in determining association preferences among *X. laevis* tadpoles as well. To compare the influence of genome-wide relatedness versus the MHC in directing association preferences in *X. laevis* tadpoles, I examine MHC-linked association preferences among non-siblings as well as between MHC-dissimilar siblings and MHC-similar non-siblings in the next chapter.

Chapter 8 : Evidence for *Xenopus laevis* Tadpole Association Preferences Associated with Genetically Determined Cues that are Unlinked to the MHC

In the previous two chapters I showed that *X. laevis* tadpoles preferentially associate with MHC-similar tadpoles when controlling for other genetic differences by using only siblings as stimulus tadpoles. In this chapter I investigate whether variation elsewhere in the genome contributes to tadpole association preferences. In two separate experiments I tested whether (a) tadpoles preferentially associate with either MHC-identical non-siblings or siblings with which they shared no MHC haplotypes and (b) tadpoles preferentially associate with unrelated MHC-identical tadpoles over unrelated tadpoles with which they shared no MHC haplotypes. I found (a) no significant association preferences when tadpoles were presented with a choice between either MHC-identical non-siblings or MHC-different siblings. (b) Among tadpoles from a different sibship, subjects demonstrated a significant association preference for MHC-different tadpoles over MHC-identical tadpoles. Furthermore, subjects moved between non-sibling stimulus groups significantly fewer times than did subjects from chapter 6 between sibling stimulus groups. These results demonstrate that there are other genetically determined cues, unlinked to the MHC, that also contribute to tadpole association preferences.

INTRODUCTION

In chapter 6 I found that premetamorphic *X. laevis* tadpoles homozygous at the MHC preferentially associate with MHC-identical siblings over siblings with which they shared no MHC haplotypes. Because all stimulus subjects were siblings in that study, I controlled for genetic variation elsewhere in the genome that might influence association preferences, thereby demonstrating that *X. laevis* tadpoles discriminate between conspecifics based on MHC similarity. Nonetheless, since I had controlled for non-MHC-linked cues, the findings cannot preclude the possibility that other genes contribute to the cues that direct association preferences in tadpoles and potentially facilitate kin recognition. Furthermore, if *X. laevis* tadpoles discriminated kin by MHC-type alone, the 25 % of full siblings that share no MHC haplotypes with an individual would not be recognized as kin. Reliable kin recognition would therefore depend not only on the MHC, but also on other parts of the genome.

Hughes and Hughes (1995) point out that MHC-associated preferences, such as those found in *X. laevis* tadpoles (Chapters 6 and 7), may result from an 'innate' tendency to associate based on relatedness which is inferred from cues including, but not limited to, the MHC. There are several choice test designs that may be employed to test the hypothesis that the MHC genes are responsible for tadpole association preferences in concert with other parts of the genome. One might test association preferences between siblings and non-siblings that are all MHC-identical, thereby controlling for MHC-directed association preferences. This is, however, beyond the scope of this thesis as the subjects with known MHC haplotypes used here are from an inbred laboratory colony with limited genetic diversity. A lack of observable association preference may result from the limited genetic diversity in these laboratory animals. Hughes and Hughes (1995) suggest the following experimental design as a 'straightforward' way of deciding between the hypotheses that MHC-associated preferences are a function of MHC-determined cues alone or of kin discrimination. Each of a number of test subjects would be given a choice between cues from full siblings with which they shared no MHC haplotype and cues from unfamiliar, unrelated individuals with which they shared

both MHC haplotypes. Rajakaruna *et al.* (2006) found that sibships of Atlantic salmon (*Salmo salar*) and brook trout (*Salvelinus fontinalis*) can discriminate MHC-identical siblings from MHC-different siblings, MHC-identical non-siblings from MHC-different siblings, but not MHC-identical non-siblings from MHC-different siblings.

In the current study, I investigate whether variation in the genome unlinked to the MHC contributes to *X. laevis* tadpole association preferences following the choice test design recommended by Hughes and Hughes (1995); I tested whether tadpoles preferentially associate with either MHC-identical non-siblings or MHC-disparate siblings. Since *X. laevis* tadpoles at premetamorphic developmental stages preferentially associate with siblings over non-siblings (chapter 5) and with tadpoles with which they share a greater MHC-similarity (chapters 6 and 7), a preference for either MHC-identical non-siblings or MHC-different siblings should provide insight into the role of non-MHC genetic differences in directing association preferences. An association preference for MHC-identical non-siblings would indicate that non-MHC genetic differences contribute little to tadpole association preferences, whereas an association preference for MHC-different siblings would indicate that the MHC contributes a relatively minor role in directing tadpole association preferences. A lack of an association preference for either MHC-identical non-siblings or MHC-disparate siblings would indicate that both MHC and non-MHC genetic differences contribute to the cues involved in tadpole discrimination.

I also repeat the study presented in chapter 6 using stimulus tadpoles from a different parental cross rather than the same parental cross as the subject tadpoles. I tested whether tadpoles preferentially associate with MHC-identical or MHC-different stimulus tadpoles from a different sibship. An association preference for MHC-identical non-siblings would indicate that relatedness between tadpoles does not influence MHC-biased association preferences. If MHC-linked association preferences differ among non-siblings from those found among siblings in chapter 6, relatedness of the stimulus tadpoles would appear to affect MHC-biased association preferences.

I examine movements of subjects among non-sibling stimulus groups and compare these to those of subjects tested among sibling stimulus groups. If there are any differences in MHC based association preferences between these two test conditions, associated behavioural responses to the presence of siblings or non-siblings may also be reflected in subject activity.

METHODS

Using the genotyping methods and association preference choice test procedure described in chapter 4 (Methods), I tested whether (a) premetamorphic tadpoles preferentially associate with MHC-identical non-sibling stimulus tadpoles or with MHC-different sibling stimulus tadpoles, or (b) premetamorphic tadpoles preferentially associate with MHC-identical or MHC-different tadpoles with both groups arising from a different family. For both experiments I used progeny from *rg*×*rg*, *fg*×*fg*, and *fr*×*fr* crosses using the same parental frogs used in chapters 6 and 7.

MHC-identical non-siblings vs. MHC-different siblings

In the first experiment I tested whether tadpoles prefer to associate with MHC-identical non-siblings over siblings with which they shared no MHC haplotypes (i.e. do *rr* (*rg*×*rg* sibship) prefer *rr* (*fr*×*fr* sibship) over *gg* (*rg*×*rg* sibship)). Sample sizes of 6 replicate test groups are given in Table 8.1 along with subject and stimulus genotypes and sibships.

I evaluated the effect of MHC similarity on tadpole association preference by hierarchically nested analysis of variance using type III sum of squares (Hill & Lewicki 2006). I compared alternate subjects for the time spent associating with MHC-identical non-siblings and that spent associating with MHC-dissimilar non-siblings. The effects of MHC similarity, family nested within MHC similarity, and genotype nested within family and MHC similarity were included in the analysis of variance. I compared the number of subjects

that spent more time on the side of the tank near the MHC-identical stimulus groups to the number of subjects that spent more time on the side of the tank near the MHC-different stimulus groups using the binomial distribution with Bonferroni adjusted alpha levels of 0.0083 (0.05/6) per test.

MHC-identical non-siblings vs. MHC-disparate non-siblings

In the second experiment I tested whether MHC-homozygous tadpoles prefer to associate with MHC identical non-siblings over non-siblings with which they shared no MHC haplotypes (i.e. do *rr* (*rg*×*rg* sibship) prefer *rr* (*fr*×*fr* sibship) over *ff* (*fr*×*fr* sibship)). Sample sizes of 6 replicate test groups are given Table 8.3 along with subject and stimulus genotypes and sibships.

I evaluated the effect of MHC similarity on tadpole association preference by hierarchically nested analysis of variance using type III sum of squares (Hill & Lewicki 2006). I compared alternate subjects for the time spent associating with MHC-identical non-siblings and that spent associating with MHC-dissimilar non-siblings. The effects of MHC similarity, family nested within MHC similarity, and genotype nested within family and MHC similarity, were included in the analysis of variance. To determine whether particular genotypes differed in their MHC similarity based association preferences, I further partitioned sum of squares by pairs of genotypes using orthogonal contrasts (see Table 8.2). I compared the number of subjects that spent more time on the side of the tank near the MHC-identical stimulus groups to the number of subjects that spent more time on the side of the tank near the MHC-different stimulus groups using the binomial distribution with Bonferroni adjusted alpha levels of 0.0021 (0.05/24) per test.

Since I used progeny from the same three pairs of parental frogs in which progeny demonstrated significant MHC-based association preferences among siblings (chapter 6) to test MHC-based association preferences among non-siblings, I investigated whether the

presence of non-sibling stimulus groups caused subjects to move more or less than when in the presence of sibling stimulus groups. I examined whether there were differences in the number of times subjects moved between the MHC-disparate stimulus groups among non-siblings as compared to the number of times subjects moved between the MHC-disparate stimulus groups among siblings (chapter 6 data set). I counted the mean number of times tadpoles crossed the centre line of the test apparatus for each subject group tested both among siblings and among non-siblings and compared differences based on subject groups and relatedness of stimulus groups by a fully crossed analysis of variance.

All data were initially tested to ensure that they satisfied assumptions of normality. Statistical inferences were based on two-tailed distributions. Analyses were computed using Statistica 7.1 (Statsoft, Tulsa, OK, USA).

RESULTS

MHC-identical non-siblings vs. MHC-different siblings

Association preferences were inconsistent between subject groups (Table 8.1) and overall subjects did not associate preferentially with MHC-identical non-siblings or MHC-different siblings ($F_{1, 101} = 1.18$; $P = 0.28$, Table 8.2). MHC-similarity based association preferences differed significantly between genotypes within families ($F_{6, 101} = 2.24$; $P = 0.045$), but this is be due to the strong tendency of only *ff* tadpoles from the *fg*×*fg* parental to spend more time near MHC-identical non-siblings than near MHC-disparate siblings (Table 8.1). There were no significant differences in any of the subject groups between the number of tadpoles that spent more time associating with MHC-identical non-siblings and the number of tadpoles that spent more time associating with MHC-different non-siblings (Table 8.1).

Table 8.1. Stimulus association preference tests among MHC-identical non-siblings and MHC-dissimilar siblings.

Stimulus MHC-types*	Subject MHC-type*	*N*	Association preference (s) for MHC-similar stimulus (Mean ± SE)**	Number of individuals		*P*†
				MHC-identical non-siblings	MHC-dissimilar siblings	
rr (*fr*) vs. *gg* (*rg*)	*rr* (*rg*)	19	-126.5 ± 177.1	9	10	0.824
gg (*fg*) vs. *rr* (*rg*)	*gg* (*rg*)	23	100.6 ± 242.5	14	9	0.308
ff (*fr*) *vs. gg* (*fg*)	*ff* (*fg*)	15	908.9 ± 240.9	12	3	0.021
gg (*rg*) vs. *ff* (*fg*)	*gg* (*fg*)	18	-275.1 ± 269.0	7	11	0.359
ff (*fg*) *vs. rr* (*fr*)	*ff* (*fr*)	16	31.0 ± 272.9	5	11	0.143
rr (*rg*) vs. *ff* (*fr*)	*rr* (*fr*)	22	-20.3 ± 206.4	10	12	0.678

*Parental line MHC-types are indicated in brackets.

**Positive means indicate preference for MHC similar and negative means indicate preference for MHC dissimilar

†Binomial test

Table 8.2. Analysis of variance for MHC similarity versus siiblings

Source of variation	df	Mean squares	*F*	*P*
MHC similarity	1	3.33×10^5	1.175	0.281
Family(MHC similarity)	4	1.80×10^5	0.633	0.640
Genotype(Family(MHC similarity))	6	6.35×10^5	2.242	0.045
Residual error	101	2.83×10^5	—	—

MHC-identical non-siblings vs. MHC-disparate non-siblings

In five of six tests, tadpoles spent more time associating with MHC-disparate non-siblings than with MHC-identical non-siblings (Fig 8.1). Overall, subjects preferentially associated with MHC-different non-siblings over MHC-identical non-siblings ($F_{1, 113} = 5.36$, $P = 0.022$; Table 8.3). MHC-similarity based association preferences did not differ significantly between subject families or between the different genotypes within families. When either *gg* or *rr* genotypes were omitted from the analysis in orthogonal contrasts, there was no significant association preference for MHC-dissimilar non-siblings over MHC-similar non-siblings. However, when the *ff* subject genotype was omitted from the analysis in an orthogonal contrast, there was a significant association preference for MHC-different non-siblings over MHC-identical non-siblings ($F_{1, 113} = 6.90$, $P = 0.010$; Fig. 8.3). This

demonstrates that *rr* and *gg* tadpoles spent more time associating with MHC-different non-siblings than did *ff* tadpoles.

There were no significant differences in any of the subject groups between the number of tadpoles that spent more time associating with MHC-identical non-siblings and the number that spent more time associating with MHC-different non-siblings (Table 8.4). These results contrast to the association preferences for MHC-identical siblings over MHC-different siblings discussed in chapter 6.

Table 8.3. Analysis of variance for choice tests among non-siblings

Source of variation	df	Mean squares	*F*	*P*
MHC similarity	1	1.66×10^{6}	5.358	0.022
Family(MHC similarity)	4	9.00×10^{4}	0.291	0.883
Genotype(Family(MHC similarity))	3	3.72×10^{4}	0.121	0.729
Orthogonal contrasts:				
ff & *rr* - MHC similarity contrast	1	7.02×10^{5}	2.272	0.135
ff & *gg* - MHC similarity contrast	1	6.95×10^{5}	2.250	0.136
rr & *gg* - MHC similarity contrast	1	2.13×10^{6}	6.903	0.010
Residual error	113	3.09×10^{5}	—	—

Table 8.4. Binomial distributions of individual preferences

Stimulus MHC-types*	Subject MHC-type*	*N*	Number of individuals spending more time near		*P*
			MHC-identical non-siblings	MHC-dissimilar non-siblings	
ff (*fr*) *vs. rr* (*fr*)	*rr* (*rg*)	17	7	10	0.481
ff (*fr*) *vs. rr* (*fr*)	*ff* (*fg*)	16	10	6	0.332
gg (*rg*) vs. *rr* (rg)	*gg* (*fg*)	17	5	12	0.096
gg (*rg*) vs. *rr* (rg)	*rr* (*fr*)	26	9	17	0.122
ff (*fg*) *vs. gg* (*fg*)	*ff* (*fr*)	35	18	17	0.868
ff (*fg*) vs. *gg* (fg)	*gg* (*rg*)	14	6	8	0.607

*Parental MHC-types are indicated in brackets.

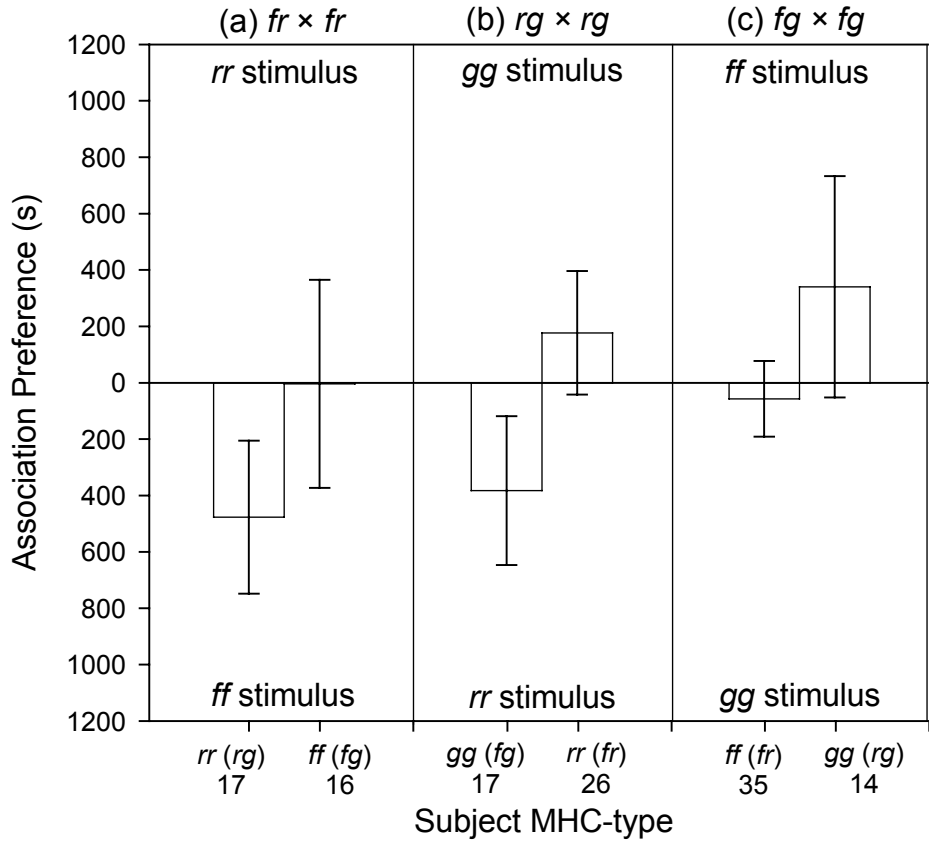

Figure 8.1: Association preferences (time spent near MHC-identical stimulus group minus time spent near MHC-different stimulus group) of subjects among non-siblings from three families (a-c). All subject genotypes, except for *ff* tadpoles from the *fg* x *fg* parental cross, tended to spend more time associating with MHC-dissimilar non-siblings than with MHC-identical non-siblings. However, none of these tendencies are significant (P>0.05; t-tests, two-tailed). Means ± SE are shown.

Tadpoles in choice tests among non-siblings crossed the centre line significantly fewer times than did tadpoles in choice tests among siblings ($F_{1, 337} = 10.14$, $P = 0.002$; Fig. 8.2; Table 8.5). Tadpoles of the different subject groups also differed significantly in the number of times they crossed the centre line ($F_{5, 337} = 12.88$, $P < 0.001$). However, the relative differences between subject groups in the number of times subjects moved between stimulus groups did not differ significantly when flanked by non-sibling or by sibling stimulus groups ($F_{5, 337} = 1.43$, $P = 0.24$).

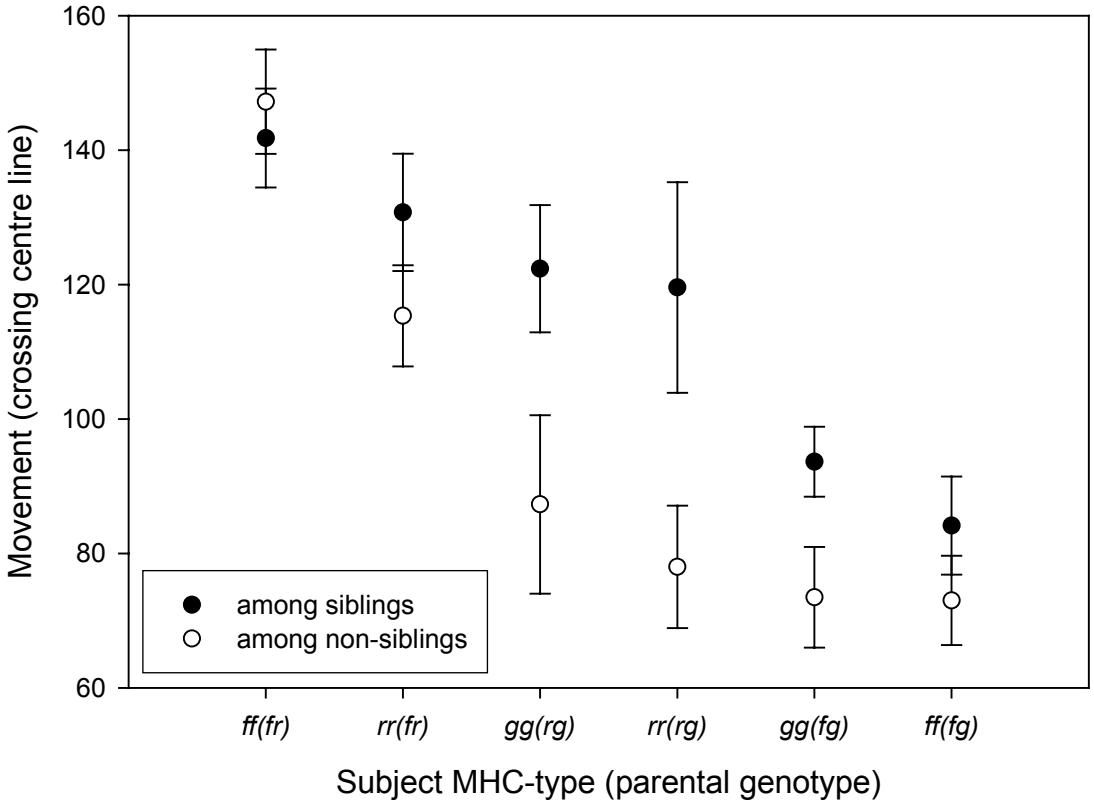

Figure 8.2: Mean number of times (± SE) that subjects crossed the centre line between the stimulus groups when flanked by MHC-disparate siblings (solid circles) or by MHC-disparate non-siblings (open circles). Both genotype, nested within sibship, and the kinship of the stimulus animals significantly influenced tadpole movement between the stimulus groups ($P < 0.001$; F-test).

Table 8.5. Analysis of variance of subject movements

Source of variation	df	Mean squares	F	P
Stimulus Relatedness	1	2.89×10^4	10.144	0.002
Subject group	5	3.67×10^4	12.876	< 0.001
Stimulus Relatedness × Subject group	5	4.06×10^3	1.425	0.215
Residual error	337	2.85×10^3	—	—

DISCUSSION

Genetically determined cues, unlinked to the MHC, contribute to association preferences in *X. laevis* tadpoles. MHC-similarity based association preferences, as found in chapter 6, seem to have been confounded by other cues associated with relatedness. In the first experiment there was no consistent difference in association preference based on genome-wide genetic similarity (MHC-dissimilar) as compared to MHC-similarity (non-siblings). The inability of tadpoles to discriminate between siblings sharing no haplotypes and MHC-identical non-siblings indicates that the rest of the genome is as potent as the MHC in determining distinguishable cues. In the second experiment, MHC-based association preferences differed among non-siblings as compared to among siblings in chapter 6. In contrast to the MHC-assortative association preferences found among sibling stimulus tadpoles in chapter 6, subjects in this experiment demonstrated a significant association preference for MHC-dissimilar non-sibling stimulus tadpoles over MHC-similar non-sibling stimulus tadpoles. Furthermore, subjects moved between stimulus groups significantly fewer times when flanked by non-sibling stimulus tadpoles as compared to subjects flanked by sibling stimulus tadpoles. Subjects exposed to sibling and non-sibling stimulus groups differed both in their MHC-linked association preferences and their movements.

The difference in MHC-linked association preferences based on the presence of siblings or non-siblings suggests that there are differences in behavioural responses to the MHC-similarity of siblings and of non-siblings. One of the major hypotheses on the origin of cues that facilitate MHC type discrimination posits that they are composed of the MHC restricted pool of peptides of 9 amino acids (9-mers) that sit in the peptide binding groove of MHC molecules and have thus been protected from exonucleolytic degradation processes (Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005). These 9-mer peptides are typically used in MHC presentation (Rammensee *et al.* 1999; Burroughs *et al.* 2004; Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005) and may be determined by differences in the types of protein sequences that different MHC haplotypes restrict, and by genomically determined differences in the

pool of endogenously derived protein sequences that the particular MHC molecules restrict. This could lead unrelated MHC-identical individuals to produce different pools of MHC restricted peptides. Thus, the MHC may not only facilitate MHC-type discrimination, but also the discrimination of genomic differences.

Such discrimination processes may account for the unexpected association preferences observed in this study. Tadpoles in the first experiment may have discriminated both differences in the pool of 9-mer peptides restricted by the dissimilar haplotypes of siblings and differences in endogenously derived proteins restricted by the shared haplotypes of non-siblings. Furthermore, if the MHC mediates MHC-type recognition processes, the discrimination of differences in MHC determined 9-mer peptides from unrelated MHC-similar conspecifics might be stronger than of those from unrelated MHC MHC-dissimilar conspecifics. If so, tadpoles in the second experiment may have preferred the presence of MHC-different non-siblings to MHC-identical non-siblings as the differences in the pool of peptides restricted by MHC-identical non-siblings were more easily discriminated.

If the differential responses to MHC similarity among siblings and among non-siblings result from a discrimination process distinct from MHC type discrimination, these findings may reflect differences in fitness consequences from associating with MHC-similar siblings versus MHC-similar non-siblings. There may be greater fitness costs in associating with MHC-similar non-siblings than with MHC-similar siblings or greater fitness benefits in associating with MHC-similar siblings than with MHC-similar non-siblings. These may be factors that influence the different contexts to which tadpoles respond differently with regards to MHC-linked association preferences.

Certain haplotypes, such as *f*, may be more attractive regardless of MHC similarity. Whereas *ff* tadpoles tended to spend equal amounts of time associating with MHC-identical and MHC-different non-siblings, *rr* and *gg* tadpoles demonstrated a stronger association preference for MHC-different non-siblings than for MHC-identical non-siblings, regardless

of the dissimilar MHC haplotype, This is interesting in light of the fact that *rr* and *gg* tadpoles are more susceptible than *ff* tadpoles to the common bacterium, *Aeromonas hydrophila*, in laboratory experiments (Barribeau 2007). Furthermore, *ff* tadpoles are more susceptible to the ranavirus, frog virus 3 (FV3), than are *jj* tadpoles (Gantress *et al.* 2003). Unfortunately, *rr* and *gg* tadpoles were not tested for susceptibility to FV3 (Gantress *et al.* 2003). It may be that only individuals that are susceptible to ubiquitous pathogens, such as *A. hydrophila* and ranavirus, avoid MHC-similar tadpoles among non-siblings to reduce their exposure to such a ubiquitous pathogen or to locally adapted strains of it.

The difference in number of times tadpoles crossed the centre line of the test apparatus in the presence of siblings or non-siblings suggests that there are differences in movement or dispersal associated with the presence of siblings or non-siblings, or even otherwise genetically variable conspecifics. Although replicate subject groups also differed significantly in the number of times subjects moved between stimulus groups, the relative differences in movement between the two data sets (among siblings vs. among non-siblings) were consistent. Thus, the movement differences between subject groups seem to be due to heritable differences in behaviour. Since the experiments were not conducted during the same time frame (different years), one must be careful not to over-interpret the differences in movements based on the presence of siblings or non-siblings. The differences may have resulted from other temporally determined factors.

To obtain a better understanding of the relative influence of genetic variation other than that of the MHC on tadpole association preferences as compared to that of the MHC itself, one would have to examine association preferences between siblings and non-siblings while controlling for differences at the MHC using tadpoles bred from wild caught frogs with naturally occurring genomic differences. More genetically variable laboratory strains are required to further investigate the role of genes unlinked to the MHC in directing tadpole association preferences. In his thesis, David Bos argues that developing single stranded conformational polymorphism (SSCP) MHC-typing methods for outbred frogs would be

unsuitable for such a study because SSCP's from distinct MHC alleles may have very similar migration patterns on electrophoretic gels (Bos 2005). However, in combination with sequence confirmation of parental frogs, this problem can be easily avoided.

Chapter 9 : General Discussion

The central objectives of this thesis were to investigate kin recognition abilities in *Xenopus laevis* tadpoles and determine whether the major histocompatibility complex (MHC) can facilitate kin recognition in *X. laevis*. I found that *X. laevis* tadpoles preferentially associate with kin at developmental stages before Thyroid hormone (TH) dependent development. At TH-induced developmental stages (approaching metamorphosis), however, I found that this kin association preference switches to an association preference for non-kin. At developmental stages in which tadpoles preferentially associate with kin, I also found that tadpoles preferentially associate with MHC-similar siblings over MHC-dissimilar siblings. Furthermore these MHC-linked association preferences among siblings correlate with shared numbers of amino acids in the peptide binding region (PBR) of the MHC, suggesting that the MHC-PBR is responsible for MHC type discrimination. However, tadpoles did not discriminate between MHC-similar non-siblings and MHC-dissimilar siblings, and they preferentially associated with MHC-dissimilar non-siblings over MHC-similar non-siblings. Thus tadpole association preferences must be determined by MHC similarity in concert with other genes that represent genome wide relatedness.

The MHC-assortative preferences between familiar MHC disparate siblings provide evidence for self-referent matching of MHC determined phenotypes. The tadpoles use highly polymorphic matching loci to socially discriminate among conspecifics, which should permit the effective discrimination of kin by genetic similarity detection (Grafen 1990).

In the choice tests, in which tadpoles demonstrated significant MHC-assortative association preferences (‘2 vs. 0’ and ‘2 vs. 1’), subjects were presented with stimulus groups that either bore or did not bear any MHC-dissimilar haplotypes. In contrast, in the choice tests in which tadpoles demonstrated no significant MHC-assortative association preferences (‘1 vs. 0’ and ‘2 vs. 1 – heterozygotes’), subjects were presented either with stimulus groups that both bore MHC-dissimilar haplotypes (‘1 vs. 0’) or with stimulus groups that both bore no MHC-dissimilar haplotypes (‘2 vs. 1 – heterozygotes’). Thus, MHC type discrimination

depends on variable differences in numbers of dissimilar haplotypes rather than in numbers of shared haplotypes.

The correlation between MHC-linked association preferences and shared amino acids in the PBR of the MHC provides empirical support for the hypothesis that MHC molecules, and in particular, the peptide binding groove, determine MHC-specific cues used in MHC type recognition. The peptide binding groove of MHC molecules determines the pool of 9-mer peptides cleaved from longer protein sequences (Rammensee *et al.* 1999). These 9-mer peptides are used in MHC presentation (Rammensee *et al.* 1999; Burroughs *et al.* 2004) and also have been found to influence pregnancy block in mice, (Leinders-Zufall *et al.* 2004) and mate choice in sticklebacks (Milinski *et al.* 2005). Furthermore, Atlantic salmon mating preferences (Landry *et al.* 2001) and MHC-based discriminations in mice (Carroll *et al.* 2002) also correspond to the genetic distances between MHC types as determined by amino acid differences in the PBR. In *X. laevis*, an inverse correlation has been found between the MHC-PBR similarity of frog conditioned water exposed to tadpoles and their development rates (Barribeau 2007). Thus, the preferential association with conspecifics that share greater similarity in the PBR may result in optimal developmental rates.

Since tadpoles demonstrate no association preference for either MHC-similar non-siblings or MHC-dissimilar siblings, the rest of the genome is as potent as the MHC in determining distinguishable cues. Since tadpoles demonstrated reversed MHC-linked association preference among siblings and non-siblings, genomic differences must influence MHC-linked association preferences. I speculate that genomically determined differences in protein sequences, from which MHC restricted 9-mer peptides are cleaved (Leinders-Zufall *et al.* 2004; Milinski *et al.* 2005), could lead unrelated MHC-identical individuals to produce different pools of MHC restricted peptides. Thus, the MHC may not only facilitate MHC-type discrimination, but also the discrimination of genomic differences. Furthermore, if the MHC mediates MHC-type recognition processes, the discrimination of differences in MHC

determined 9-mer peptides from unrelated MHC-similar conspecifics might be stronger than of those from unrelated MHC-dissimilar conspecifics.

At earlier developmental stages, tadpoles preferred siblings over non-siblings, and MHC similar siblings over MHC dissimilar siblings At subsequent larval developmental stages, when tadpoles undergo drastic neuroanatomical and physiological changes (Dodd & Dodd 1976; White & Nicoll 1981; Iwasawa & Yamaguchi 1984; Cohen & Kelley 1996; Kelley 1996; Robertson & Kelley 1996; Reiss & Burd 1997; Rollins-Smith *et al.* 1997; Furlow & Neff 2006), tadpoles preferentially associated with non-siblings. However, whether MHC-assortative preferences also switch to MHC-disassortative preferences at these later larval developmental stages still must be determined. During later larval developmental stages, MHC-expression changes (Rollins-Smith *et al.* 1997) and tadpoles are immunocompromised (Rollins-Smith 1998). Analogous association preferences for MHC-dissimilar tadpoles at later developmental stages may help reduce the spread of disease among more MHC-diverse conspecifics (Hamilton 1987; Shykoff & Schmid-Hempel 1991) when they become immunocompromised.

The mechanism by which tadpoles discriminate MHC-type differences may be relevant to other contexts such as mate choice or philopatry. Examining the role of MHC-linked preferences in *X. laevis* mate choice may provide insight into how MHC-biased behaviour may be involved in enhancing inbreeding avoidance, disease resistance, and the maintenance of MHC polymorphisms.

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Appendix 1: Critical Review of the Evidence for and Interpretation of Self-Referent Phenotype Matching

Self referent phenotype matching (SRPM) and genetic recognition systems are frequently discussed as distinct mechanisms of kin recognition. Most studies that focus on self-referent phenotype matching assume that since familiarity with conspecifics is not required for SRPM to occur, and that SRPM is also distinct from familiarity based recognition systems (Heth *et al.* 1998; Hauber *et al.* 2000; Mateo & Johnston 2000, 2003; Neff & Sherman 2005). There are, however, basic similarities in the ontogenetic processes underlying all of these hypothetical recognition mechanisms (Waldman 1987). Both involve the assessment of relatedness based on matching conspecifics' traits with those expected in kin. The ability to discriminate traits may be learned from prior social interactions or be internally derived (acquired from self-observation or directly determined in a genetically programmed template) (Waldman 1987). However, even when recognition is based on comparison with one's own traits, this process may very well reflect a learned familiarization of oneself or one's own traits that are most pervasive in ones environment (Blaustein 1983; Waldman 1987). In the following review of the empirical studies supporting SRPM, results can equally be interpreted as recognition being facilitated by a genetic recognition system as well as by some learned familiarity with the cues that reliably predict relatedness (whether from oneself or from conspecifics in contexts associated with kinship). Even when SRPM results from a learned familiarity with ones own phenotypic cues, this does not necessarily occur via self-inspection, but could result from the fact that ones own phenotypic traits are most pervasive in an individual's environment.

Various field studies suggest that self-referent phenotype matching is likely to facilitate kin recognition in the wild. Most of these studies have been done in lekking species in which male leks (groups) are visited by females primarily to mate (Hoeglund *et al.* 1999). Attraction of a female to a lek increases the mating potential of all individuals in a lek (Shorey *et al.* 2000). Therefore, according to kin-selection theory, both reproducing and non-

reproducing males may gain indirect inclusive fitness benefits from preferentially forming leks with closely related individuals (Petrie *et al.* 1999; Shorey *et al.* 2000). Studies in peacocks (*Pavo cristatus*) (Petrie *et al.* 1999), black grouse (*Tetrao tetrix*) (Hoeglund *et al.* 1999), red grouse (*Lagopus lagopus scoticus*) (Piertney *et al.* 1999), and white-bearded manakins (*Manacus manacus*) (Shorey *et al.* 2000) have demonstrated that leks are composed of clusters of closely related individuals. In peacocks, such kin-leks (determined by multilocus DNA fingerprints) are most likely to result from self-referent phenotype matching since observed leks were composed of individuals that hatched in captivity among eggs of non-relatives before they were released (Petrie *et al.* 1999). Kin (determined by DNA microsatellites) discrimination in the white-bearded manakins is also likely to be a function of self-referent phenotype matching, because their clutch sizes of only one or two eggs are proposed to be too small for social learning of familial characteristics (Shorey *et al.* 2000; Hauber and Sherman 2001). Hauber and Sherman (2001) suggest that the kin-leks (determined by DNA microsatellites) in the black grouse (Hoeglund *et al.* 1999) and in the red grouse (Piertney *et al.* 1999) are also a result of self-referent phenotype matching because broods dissolve and siblings cease to interact more than a year before males matured sexually and developed male plumage, displays, and vocalizations (Piertney *et al.* 1999; Hauber & Sherman 2001)**.**

In wild baboons (*Papio cynocephalus*), even though paternal half-siblings (determined by DNA microsatellites) were not less likely to consort than non-kin, paternal half-siblings exhibited lower levels of affiliative and sexual behaviour during sexual courtship than non-kin (Alberts 1999). These findings are suggestive of phenotype matching based on paternal traits (Alberts 1999). However, individuals that were born on the same troop within two years of each other were less likely to consort with each other than individuals that differed more in age, and were less affiliative and sexual when they did consort (Alberts 1999). Since age cohorts are likely to represent paternal sibships (Altmann 1979; Alberts 1999), age proximity may be an alternative social cue for paternal relatedness (Alberts 1999).

Experimental manipulation by Hauber *et al.* (2000) of brown-headed cowbird (*Molothrus ater*) feather colours with a black sharpie pen influenced association preferences among conspecifics. In simultaneous choice trials, juvenile cowbirds approached more quickly and associated for longer durations with individuals that were coloured similar (dyed or normal-coloured) to themselves. These findings are suggestive of self-referent phenotype matching as the cowbirds seem to incorporate their own plumage colour into their recognition template. This type of recognition mechanism is relevant to the life cycle of the brown-headed cowbird as it is an obligate brood parasite in which chicks are typically reared among heterospecifics.

However, certain methodological aspects of the study limit the interpretation of the data. Assortative association preferences were only found after successive trials. Prior interactions with stimulus cowbirds, that may have behaviourally discriminated the test subjects based on their plumage during earlier trials, may therefore have influenced subsequent association preferences of the test subjects. Even though Hauber et al. (2000) could find no differences in the rate at which stimulus cowbirds directed behaviour toward dyed and control juveniles (eg., rates of pecking, head-down, and head-up displays) (Hauber & Sherman 2003), effects of other more subtle interactions between subjects and stimulus birds during earlier trials cannot be discounted.

Furthermore, brown-headed cowbirds are sexually dimorphic. The feathers of adult males are darker in pigment than those of females (Hare *et al.* 2003). Since only adult females, either normal 'female' coloured or blackened (male-like), were used as stimulus birds in the choice tests (Hauber *et al.* 2000), the results of Hauber et al. (2000) can equally be interpreted as an attraction to adults of the opposite sex (Hare *et al.* 2003). Female subjects, regardless of their plumage manipulation, spent more time associating with blackened (male-like) stimulus birds (Hauber *et al.* 2000). Undyed male subjects spent more time associating with undyed female stimulus birds, and dyed male subjects showed no

association preferences for either of the stimulus birds (Hauber *et al.* 2000). Results are very similarly confounded for approach rates.

To disentangle the effects of relatedness and prior association infants can be taken from their genetic parents and reared by unrelated foster parents (Mateo & Holmes 2004). Mateo and Johnston (2000) claim to be the first to implement such cross-fostering techniques experimentally to demonstrate in vertebrates the use of an individuals' own phenotype for kin-recognition purposes without prior experience with kin. They found that cross-fostered golden hamsters (*Mesocricetus auratus*) (males and females) approached flank-gland odours from the opposite sex of un-familiar non-kin significantly faster than flank-gland odours of familiar non-kin foster siblings or of unfamiliar kin (Mateo & Johnston 2000). Female hamsters also investigated male flank-gland odours of unfamiliar non-kin for significantly longer durations than those of familiar non-kin foster siblings or of unfamiliar kin (Mateo & Johnston 2000). Male odours from unfamiliar non-kin also elicited fewer flank marking responses by females than odours from unfamiliar kin (Mateo & Johnston 2000). There were, however, no differences in approach rates, investigation times, or scent mark responses to flank-gland odours of non-kin foster-siblings and unfamiliar kin (Mateo & Johnston 2000). Heth *et al.* (1998), however, did find that cross-fostered male hamsters demonstrated significantly greater scent marking responses to unrelated males than to related males, regardless of whether they were reared with the odour donors (Heth *et al.* 1998).

Unlike the study by Mateo and Johnston (2000), in which single hamster pups were cross-fostered in litters with two foster siblings each, the pups in the study by Heth *et al.* (1998) were cross-fostered together with one biological sibling and two foster siblings. As a result, kin-odours contributed to half of the odours in the litters used in the Heth *et al.* (1998) study, while kin odours contributed to a third of the odours in the litters used in the Mateo and Johnston (2000) study in which each foster pup had only itself as a source of information about how genetic relatives smell. The greater familiarity with kin odours in the Heth *et al.*

(1998) study may account for the greater response to kin-odours over familiar non-kin odours as compared to the Mateo and Johnston (2000) study.

Mateo and Johnston (2000) also tested the differential responses of female hamsters to odours of unfamiliar sisters and sisters of foster-siblings. The odours from biological sisters were investigated for longer durations than those from the unfamiliar sisters of their foster siblings. Mateo and Johnston (2000) conclude from these results that self-odours were weighted more heavily in their templates than those of their foster family, even though stronger responses would be expected to be directed to non-kin odours if this were the case (Heth *et al.* 1998; Todrank & Heth 2001). Since flank mark odours elicit a greater flank marking response from unfamiliar non-siblings than from familiar biological siblings (Heth *et al.* 1998), Mateo and Johnston's (2000) results indicate that odours of their foster family were weighted more heavily in their templates than self-odours.

Another confounding variable in both the Mateo and Johnston (2000) study and the Heth *et al.* (1998) study is that subjects were separated at 30 days of age and housed individually until they were used in experiments 10-30 days (Mateo & Johnston 2000) or 2-3 months later (Heth *et al.* 1998). The observed kin-odour biases may therefore have been a function of familiarity with ones own odour (which are more similar to kin-odours) to which they were solely exposed to during their pre-testing period of isolation rather than of self-referencing phenotype matching during the pre-weaning period in cross-fostered litters. This is especially relevant when considering responses to scent marks because hamsters' flank glands do not begin secreting until pups are more than one month old (Hauber & Sherman 2000). Post-partum familiarization with close kin during the hours prior to cross-fostering may have similarly contributed to the observed kin-odour biases observed by Heth *et al.* (1998) and Mateo and Johnston (2000) (Hare *et al.* 2003).

Parental male bluegill sunfish (*Lepomis macrochirus*) are able to distinguish between water sources that have been conditioned by their own offspring versus unrelated fry (Neff &

Sherman 2005). Neff and Sherman (2005) purport that these results imply the use of self-referent phenotype matching for kin recognition because the only referents available to the individually housed experimental subjects were cues emanating from their own body (Neff & Sherman 2005). However, Hauber and Sherman (2003) point out the difficulty of unequivocally demonstrating self-referent phenotype matching when experimentally attempting to eliminate all experiences of an individual with kin odours by placing subjects into isolation, as isolated subjects will then be solely exposed to self-odours that are representative of kin (Hauber & Sherman 2003).

Olsen *et al.* (1998) were able to circumvent problems associated with rearing test subjects in isolation by testing for self-referent MHC-genotype matching odour preferences among juvenile arctic charr siblings (*Salvelinus alpinus*) with which they were reared in groups. In a fluvarium, subjects spent more time in the chamber scented by a full siblings whose MHC genotype was identical to their own than in the chamber scented by full siblings whose MHC genotype was different (Olsen *et al.* 1998). Unfortunately, there is apparent experimental bias in the manner the data were collected. Based on control tests, in which both sides of the fluvarium contained plain water, they subsequently placed the fish predicted to be the preferred one in the least preferred aquarium (Olsen *et al.* 1998). The authors claim that this would have a conservative effect on the observed preferences (Olsen *et al.* 1998). However, in their analyses they compared the times spent in the designated "least preferred aquarium" between the subjects in the control tests that spent less time in that aquarium and the subjects in the sibling odour tests that spent more time in that aquarium (Olsen *et al.* 1998). As a result their methods were not conservative at all, but rather exaggerated the observed preferences.

Aeschlimann et al. (2003) found that female three-spined sticklebacks (*Gasterosteus aculeatus*) choose mates to achieve an immunologically optimum number of MHC alleles for their offspring. Their research demonstrates that female sticklebacks use information about their own MHC polymorphisms to determine the optimal MHC polymorphisms of potential

mate's to complement their own set of alleles (Aeschlimann *et al.* 2003). This presents a clear example by which self-referencing facilitates a genetic recognition system.

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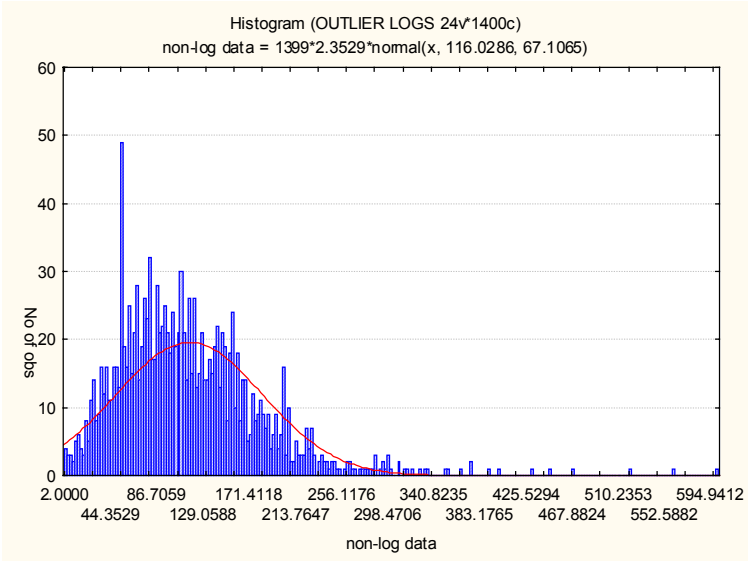
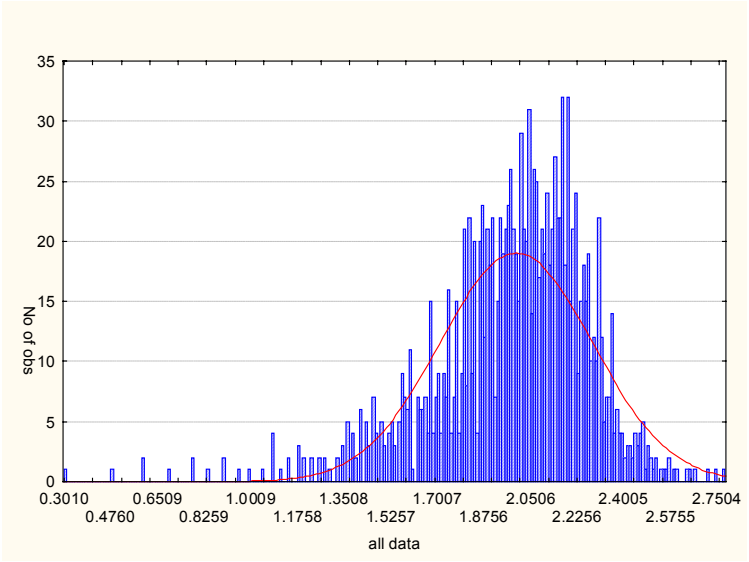
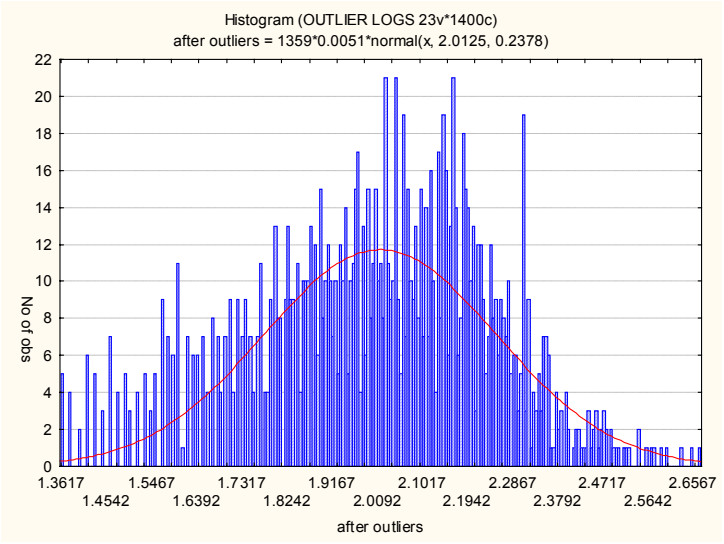
Appendix 2: Statistical Figures and Tables

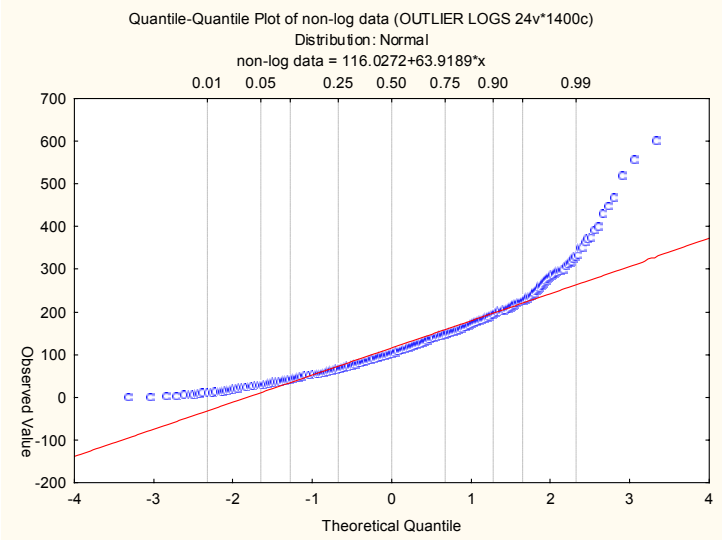
Appendix 2a: Histograms of overall movement data before (top) and after log transformations (middle) and after removal of outliers (bottom).

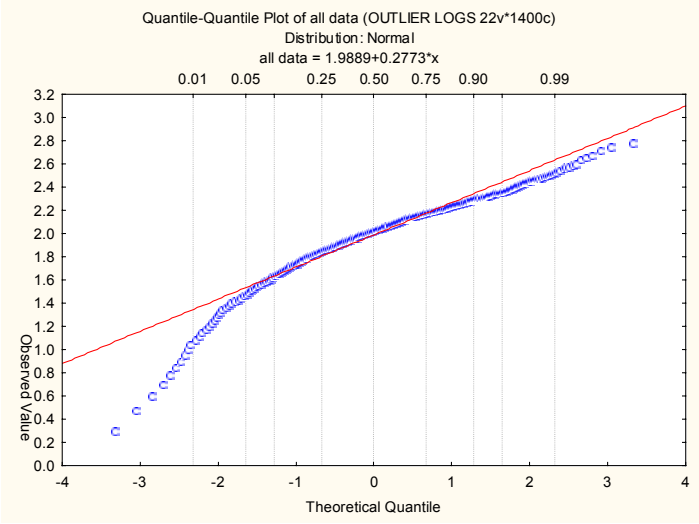


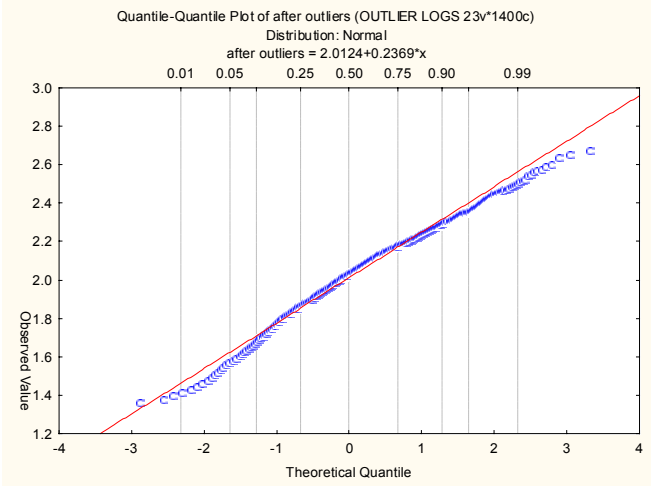




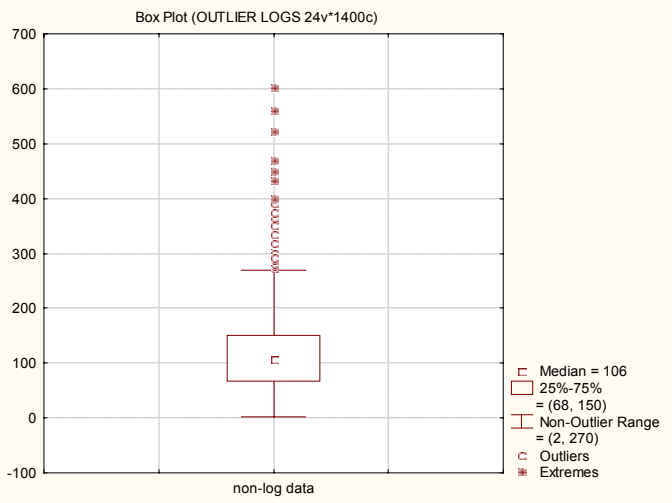
Appendix 2b: Quantile-quantile (normality) plots of raw overall movement data before (top) and after log transformations (middle) and after removal of outliers (bottom).

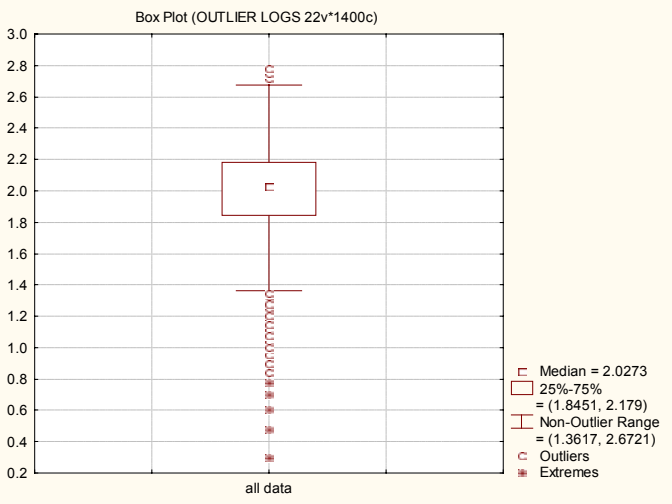


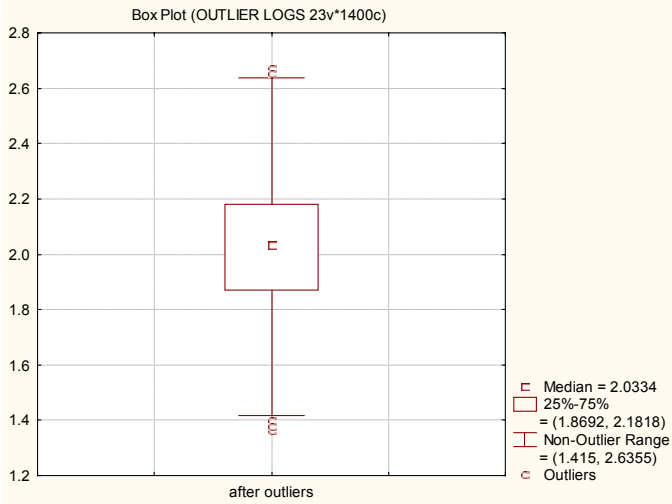




Appendix 2c: Box plots of overall movement data before (top), after log transformations (middle), and after removal of outliers (bottom). Removed outliers (2.86 %) are indicated in the log transformed plot (middle). Out of 1399 subjects, 37 subjects which crossed the centre line ≤22 times and 3 subjects which crossed the center line ≥470 times were subsequently removed.







		Shared class I PBR amino acid residues		Shared class II PBR amino acid residues		Shared class I and II PBR amino acid residues	
Subject	Stimulus	Proportion per haplotype	%	Proportion per haplotype	%	Proportion per haplotype	%
jj	*jj*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
jj	*rr*	134/181 + 134/181	74.033	320/356 + 320/356	89.888	454/537 + 454/537	84.544
jj	*rj*	134/181 + 181/181	87.017	320/356 + 356/356	94.944	454/537 + 537/537	92.272
rr	*jj*	134/181 + 134/181	74.033	320/356 + 320/356	89.888	454/537 + 454/537	84.544
rr	*rr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rr	*rj*	181/181 + 134/181	87.017	356/356 + 320/356	94.944	537/537 + 454/537	92.272
rj	*jj*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rj	*rr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rj	*rj*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
gg	*gg*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
gg	*rr*	140/181 + 140/181	77.348	324/356 + 324/356	91.011	458/537 + 458/537	85.289
gg	*rg*	140/181 + 181/181	88.674	324/356 + 356/356	95.506	458/537 + 537/537	92.644
rr	*gg*	140/181 + 140/181	77.348	324/356 + 324/356	91.011	458/537 + 458/537	85.289
rr	*rr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rr	*rg*	181/181 + 140/181	88.674	356/356 + 324/356	95.506	537/537 + 458/537	92.644
rg	*gg*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rg	*rr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rg	*rg*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
ff	*ff*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
ff	*gg*	146/181 + 146/181	80.663	330/356 + 330/356	92.697	476/537 + 476/537	88.641
ff	*fg*	146/181 + 181/181	90.331	330/356 + 356/356	96.348	476/537 + 537/537	94.320
gg	*ff*	146/181 + 146/181	80.663	330/356 + 330/356	92.697	476/537 + 476/537	88.641
gg	*gg*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
gg	*fg*	181/181 + 146/181	90.331	356/356 + 330/356	96.348	537/537 + 476/537	94.320
fg	*ff*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
fg	*gg*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
fg	*fg*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
ff	*ff*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
ff	*rr*	156/181 + 156/181	86.188	337/356 + 337/356	94.663	493/537 + 493/537	91.806
ff	*fr*	156/181 + 181/181	93.094	337/356 + 356/356	97.331	493/537 + 537/537	95.903
rr	*ff*	156/181 + 156/181	86.188	337/356 + 337/356	94.663	493/537 + 493/537	91.806
rr	*rr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
rr	*fr*	181/181 + 156/181	93.094	356/356 + 337/356	97.331	537/537 + 493/537	95.903
fr	*ff*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
fr	*rr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000
fr	*fr*	181/181 + 181/181	100.000	356/356 + 356/356	100.000	537/537 + 537/537	100.000

Subject	Stimulus groups	MHC class I PBR			MHC class II PBR		
		% amino acid similarity of stimulus groups		Stimulus differential (%)	% amino acid similarity of stimulus groups		Stimulus differential (%)
		MHC-similar	MHC-dissimilar		MHC-similar	MHC-dissimilar	
jj	*jj* vs. *rr*	100.000	74.033	25.967	100.000	89.888	10.112
rr	*jj* vs. *rr*	100.000	74.033	25.967	100.000	89.888	10.112
jj	*jj* vs. *rj*	100.000	87.017	12.983	100.000	94.944	5.056
rr	*jj* vs. *rj*	87.017	74.033	12.983	94.944	89.888	5.056
rj	*jj* vs. *rj*	100.000	100.000	0.000	100.000	100.000	0.000
jj	*rr* vs. *rj*	87.017	74.033	12.983	94.944	89.888	5.056
rr	*rr* vs. *rj*	100.000	87.017	12.983	100.000	94.944	5.056
rj	*rr* vs. *rj*	100.000	100.000	0.000	100.000	100.000	0.000
gg	*gg* vs. *rr*	100.000	77.348	22.652	100.000	91.011	8.989
rr	*gg* vs. *rr*	100.000	77.348	22.652	100.000	91.011	8.989
gg	*gg* vs. *rg*	100.000	88.674	11.326	100.000	95.506	4.494
rr	*gg* vs. *rg*	88.674	77.348	11.326	95.506	91.011	4.494
rg	*gg* vs. *rg*	100.000	100.000	0.000	100.000	100.000	0.000
gg	*rr* vs. *rg*	88.674	77.348	11.326	95.506	91.011	4.494
rr	*rr* vs. *rg*	100.000	88.674	11.326	100.000	95.506	4.494
rg	*rr* vs. *rg*	100.000	100.000	0.000	100.000	100.000	0.000
ff	*ff* vs. *gg*	100.000	80.663	19.337	100.000	92.697	7.303
gg	*ff* vs. *gg*	100.000	80.663	19.337	100.000	92.697	7.303
ff	*ff* vs. *fg*	100.000	90.331	9.669	100.000	96.348	3.652
gg	*ff* vs. *fg*	90.331	80.663	9.669	96.348	92.697	3.652
fg	*ff* vs. *fg*	100.000	100.000	0.000	100.000	100.000	0.000
ff	*gg* vs. *fg*	90.331	80.663	9.669	96.348	92.697	3.652
gg	*gg* vs. *fg*	100.000	90.331	9.669	100.000	96.348	3.652
fg	*gg* vs. *fg*	100.000	100.000	0.000	100.000	100.000	0.000
ff	*ff* vs. *rr*	100.000	86.188	13.812	100.000	94.663	5.337
rr	*ff* vs. *rr*	100.000	86.188	13.812	100.000	94.663	5.337
ff	*ff* vs. *fr*	100.000	93.094	6.906	100.000	97.331	2.669
rr	*ff* vs. *fr*	93.094	80.663	12.431	97.331	94.663	2.669
fr	*ff* vs. *fr*	100.000	100.000	0.000	100.000	100.000	0.000
ff	*rr* vs. *fr*	93.094	80.663	12.431	97.331	94.663	2.669
rr	*rr* vs. *fr*	100.000	93.094	6.906	100.000	97.331	2.669
fr	*rr* vs. *fr*	100.000	100.000	0.000	100.000	100.000	0.000

Subject	Stimulus groups	MHC class I and II PBR		
		% amino acid similarity of stimulus groups		Stimulus differential (%)
		MHC-similar	MHC-dissimilar	
jj	*jj* vs. *rr*	100.000	84.544	15.456
rr	*jj* vs. *rr*	100.000	84.544	15.456
jj	*jj* vs. *rj*	100.000	92.272	7.728
rr	*jj* vs. *rj*	92.272	84.544	7.728
rj	*jj* vs. *rj*	100.000	100.000	0.000
jj	*rr* vs. *rj*	92.272	84.544	7.728
rr	*rr* vs. *rj*	100.000	92.272	7.728
rj	*rr* vs. *rj*	100.000	100.000	0.000
gg	*gg* vs. *rr*	100.000	85.289	14.711
rr	*gg* vs. *rr*	100.000	85.289	14.711
gg	*gg* vs. *rg*	100.000	92.644	7.356
rr	*gg* vs. *rg*	92.644	85.289	7.356
rg	*gg* vs. *rg*	100.000	100.000	0.000
gg	*rr* vs. *rg*	92.644	85.289	7.356
rr	*rr* vs. *rg*	100.000	92.644	7.356
rg	*rr* vs. *rg*	100.000	100.000	0.000
ff	*ff* vs. *gg*	100.000	88.641	11.359
gg	*ff* vs. *gg*	100.000	88.641	11.359
ff	*ff* vs. *fg*	100.000	94.320	5.680
gg	*ff* vs. *fg*	94.320	88.641	5.680
fg	*ff* vs. *fg*	100.000	100.000	0.000
ff	*gg* vs. *fg*	94.320	88.641	5.680
gg	*gg* vs. *fg*	100.000	94.320	5.680
fg	*gg* vs. *fg*	100.000	100.000	0.000
ff	*ff* vs. *rr*	100.000	91.806	8.194
rr	*ff* vs. *rr*	100.000	91.806	8.194
ff	*ff* vs. *fr*	100.000	95.903	4.097
rr	*ff* vs. *fr*	95.903	91.806	4.097
fr	*ff* vs. *fr*	100.000	100.000	0.000
ff	*rr* vs. *fr*	95.903	91.806	4.097
rr	*rr* vs. *fr*	100.000	95.903	4.097
fr	*rr* vs. *fr*	100.000	100.000	0.000