ORGANISMAL PERFORMANCE ASSAYS FOR DETECTING ADVERSE HEALTH IMPACTS OF NUTRITIONAL AND TOXIC EXPOSURES: CONSEQUENCES OF DIETARY FRUCTOSE

by

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ABSTRACT

Determining the health impacts of a nutritional regimen, suspected toxicant or other treatment is often a difficult task in both the realms of safety assessment and basic research. There are far too many examples of agents, once considered safe, found later through epidemiology (or other means) to cause adverse health effects. To prevent such experimentation on ourselves there is a great societal need for broad, sensitive assays able to detect toxicity at human-relevant exposure levels. Similarly, basic researchers often lack the experimental tools necessary to determine if a treatment adversely impacts the health of their model organism. We argue that these problems can be partially solved by using house mice in the crucible of their natural setting where they are challenged daily by the very tasks that have shaped them for millennia. Quantifying the lifelong fitness of experimentally treated animals directly competing with control individuals offers a sensitive and broad approach for detecting adverse health effects. We refer to this approach as an Organismal Performance Assay (OPA). To illustrate the effectiveness of OPAs, herein we apply them for detecting adverse health consequences of nutritional and toxic exposures. First, using OPAs we capture adverse health impacts (decreased survival, competitive ability and reproduction) from consuming 12.5% kcal of fructose; this finding now represent the lowest observed adverse effect level for dietary fructose. Next, we apply OPAs to determine if differential health impacts occur due to the consumption of one, or the other, of the two common types of added sugar, high fructose corn syrup (fructose and glucose monosaccharides) or table sugar (sucrose, which is a

disaccharide of fructose and glucose), and show that the high fructose corn syrup diet increases mortality and decreases reproduction of female mice compared to sucrose, providing the first experimental evidence that the two most common forms of caloric sweeteners have differential health impacts. Next, we use OPAs to determine if an acute exposure to 3mg/kg of amine-terminated generation seven poly amido-amine dendrimers, the current maximum tolerated dose, is actually toxic and find that it is not. Finally, to address the criticism that OPAs do not lead to the underlying mechanisms of observed organismal outcomes, we illustrate the discovery of the molecular basis of the first phenomenon revealed using OPAs, major histocompatibility complex (MHC)-based mating preferences, which is done in the context of a review paper on the role of MHC during social communication.

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CHAPTER 1

USING ORGANISMAL PERFORMANCE DURING NATURAL CHALLENGES TO DETECT ADVERSE HEALTH EFFECTS FROM ENVIORNMENTAL EXPOSURES

Abstract

Determining the health impacts of a nutritional regimen, suspected toxicant or other treatment is often a difficult task in both the realms of safety assessment and basic research. There are far too many examples of agents, once considered safe, that are found later through epidemiology to cause adverse health effects. To prevent such widespread experimentation on ourselves and other animals there is a great societal need for broad, sensitive assays able to detect toxicity at relevant exposure levels. Similarly, basic researchers often lack the experimental tools necessary to determine if a treatment adversely impacts the health of their model organism. Examples include geneticists who knock-out genes and see no phenotype, or physiologists whose treatment causes numerous changes in gene expression; although it is seldom clear if these changes are adverse. We argue that these problems can be partially solved by using classic animal models (e.g., house mice) in the crucible of their natural setting where they are challenged daily by the very tasks that have shaped them for millennia. Quantifying the

lifelong fitness (and key components thereof) of experimentally treated animals directly competing with control individuals appears to offer a sensitive and broad approach for detecting adverse health effects.

Environmental impacts on disease

The importance of the role of the social and physical environment on the induction and elucidation of human disease is well established. Examples are numerous and include, but are not limited to, the role of stress in cardiovascular disease, mortality due to heart malformations manifesting themselves during student athletics, and asthmatic conditions brought on by exercise (1-3). The question begs itself, if we know that the social and physical environment is key in both exacerbating and revealing human diseases then why do we ignore the potential influences of environment when conducting animal safety research?

Typically, animal subjects are maintained under artificial conditions that do not reflect the natural environments that forged them into existence through natural selection. In essence, to study animals in these conditions is akin to studying people in an asocial environment in which they do not exert themselves, do not encounter hardships and have *ad libitum* access to well formulated food. When a substance is declared safe, ideally it is safe while practicing a typical vigorous, stressful lifestyle, not simply safe in a setting devoid of challenge. Could the failure to provide natural or seminatural environments for our laboratory animals increase the likelihood that they provide us with misinformation concerning the adversity of experimental treatments?

When comparing concordance rates between human and animal studies a certain degree of discrepancy is to be expected, but what is the cause of this disparity? Typically

this question is answered by species-based differences in genetics and physiology, and though these differences are real it seems unlikely that they account for the entire explanation. Currently, concordance rates for pharmaceutical safety assessment between rodents and humans are 43% and when rodent plus a nonrodent models are combined concordance increases to 71% (4). Pharmaceutical concordance rates are equally split between false-negatives and false-positive and they are artificially low compared to other environmental exposures as most overtly toxic substances are not considered as potential therapeutic agents. Pharmaceutical concordance rates are arguably the best tracked, but discrepancies in concordance exist in toxicology and nutrition as well. In toxicology they are generally assumed to be approximately 80% for humans and rodents (5). When we compare animal data to that generated from human studies we have changed two major variables, one of course being the species, but the other is the environment in which that organism dwells. Therefore, if we would like to increase the power and translatability of animal research a key first step is recognizing and incorporating the role of a representative natural environment.

For decades research groups studying animal behavior, ecology and evolution have used seminatural environments in many animal model systems of biomedical importance including macaques, mice, and pigs, but few researchers have used such systems in the applied fields of biomedicine, nutrition, pharmaceutics and toxicology (6-10). There are notable examples, however, in the areas of feeding psychology and addiction (11, 12). Though there is general applicability in creating more naturalistic settings for many species, the specific alterations and considerations for any given species will be unique. Therefore, though we advocate for numerous animal models to

incorporate more realistic environments we will specifically address the house mouse (*Mus musculus*) system, which is the best developed.

Organismal performance assays

We refer to our methodology as an Organismal Performance Assay (OPA). OPAs use wild house mice in seminatural enclosures where mice treated with a potential toxicant or other experimental manipulation compete directly with control animals. OPAs achieve their sensitivity and breadth because high performance from most physiological systems is required for individual success as determined by survival, social dominance, reproduction and a variety of other components of fitness. Consequently, any health declines that reduce performance of any physiological system (e.g., cardiovascular, neurological, or metabolic) are likely to be detected by OPAs and no *a priori* assumption about the target organ or mechanism of toxicity has to be made. OPAs are defined as sensitive phenotyping approaches that use seminatural conditions to challenge the physiological performance of control and experimental animals in direct competition with each other. The relative success of control and experimental animals can be compared for any measurable components of fitness, allowing detection and quantification of any reduced performance due to treatment.

The design of OPA enclosures is based on the preference of house mice to maintain territories that include isolated, dark, nest sites that offer protection from predators and infanticidal conspecifics (13-15). OPA enclosures measure about 5m by 7m ($35m^2$), but dimensions could vary. Each pen is subdivided into six subsections by hardware cloth, which provides spatial complexity. Each subsection has food and water that is associated with a set of nest boxes in either one of four "optimal" territories, which

contain nest boxes in enclosed structures or two "suboptimal" territories with nest boxes in the open. Together, the hardware fences and the two types of nest boxes create environmental complexity in which mice establish nesting sites, territorial boundaries and social hierarchies. OPA enclosures mimic habitat and social environments experienced by mice in nature, and the population density is representative of measurements from wild populations (16).

OPAs have been previously used to quantify adverse consequences associated with cousin and sibling-level inbreeding as well as bearing the selfish genetic element known as the *t complex* (17-19). The primary cause of inbreeding depression is deleterious recessive alleles that are expressed at a higher rate in inbred individuals, and though these negative consequences have been known for centuries actual fitness effects were less clear (20). Two major studies were conducted on mice indicating that the consequences of full-sibling mating are a 10% decline in litter size (21, 22). Further studies were conducted on the surviving inbred offspring, but these mice performed similar to outbred controls. We conducted OPAs on these seemingly normal inbred progeny by competing them against outbred controls and discovered an additional 500% decline in male reproduction (18). We have repeated these experiments at the level of cousin unions and OPAs revealed that this level of inbreeding reduced male fitness by 34%, challenging clinical claims that health effects from cousin-level inbreeding are tolerable (17, 23).

Since its discovery half a century ago, the mouse *t complex* has become a textbook example of a selfish gene. Despite much success characterizing its underlying genetics and transmission distortion effects, the population dynamics of this persistent

genetic polymorphism has remained paradoxical because population frequencies are far lower than theoretical predictions would suggest (24). Thus, it is likely that some form of selection is operating against the invasion and spread of *t* haplotypes among wild mouse populations. We used OPAs to discover the missing phenotypes, which were reproductive declines in both *t* bearing males and females. These reproductive defects reduced *t* allele frequencies to 49% below transmission distortion expectations (19). In all cases above, OPAs discovered large health declines associated with treatments that had been missed for decades by researchers using conventional laboratory methods.

Applying OPAs to environmental exposures

The following chapters of this disseration represent the first application of OPAs to detect and quantify health consequences of environmental expsoures. These exposures include both nutrional and toxicological exposures. While previous OPA studies have focused on genetic treatments such as the aformintioned inbreeding and *t complex,* the application of this technigue is arugabley most needed in the fields of nutrition and toxicology, where substances once considered safe, such as asbestos, DDT, diethylstilbestrol, polycyclic aromatic hydrocarbons (from grilled meat), second-hand smoke and trans fatty acids are often found to be detrimental to health after years of human exposure (25-30). OPAs help us answer the simple but crucial question, does an exposure at a given level make a mouse sick; if OPAs had been utilized to evaluate the safety of the substances mentioned above, decades of human exposure and sickness could have been avoided.

In the second chapter, OPAs are applied to dietary fructose to determine if a human relevant exposure level decreases mouse health and performance. Though

association between human disease and fructose consumption are well established and many mechanistic aspects of fructose toxicity have been elucidated at high dose levels, no experimental characterization of adversity has been made at exposure levels that are relevant to human consumption (31-37). Using OPAs, however, we determine that fructose exposure at 12.5% kcal results in increased mortality, decreased competitive ability and decreased reproductive success. The data within this chapter now represent the lowest observed adverse effect level for dietary fructose, a level experienced by 13% of Americans (37).

The third chapter focuses on using OPAs to determine if differential health impacts are associated with eating high fructose corn syrup (HFCS) (fructose and glucose monosaccharides) or table sugar (sucrose). To date, only two published rodent studies have indicated that these sugars have different impacts; however, the studies used exposure levels far beyond human relevance, and the differences described cannot readily be concluded as adverse (38, 39). Using OPAs we capture clear evidence that an exposure modeling HFCS is more detrimental than table sugar, as females fed a diet modeling HFCS experience increased mortality and decreased reproduction.

Within the fourth chapter OPAs are applied to determine if the established maximum tolerated dose of an engineered nanomaterial is actually toxic. Amine-terminated generation seven poly amido-amine (PAMAM) dendrimers are known to be toxic to mice at 10mg/kg body mass as they cause blood coagulation and death (40). Based on this observation the maximum tolerated dose was established at 3/mg/kg. Using OPAs we demonstrate that no adverse effects due to exposure are experienced from a one-time injection at this dose.

From phenotype to mechanism

The fifth and final chapter is a published review illustrating how the initial phenotype characterized using OPAs, mating preferences associated with the Major histocompatibility complex (MHC), has helped lead to the discovery of the underlying molecular mechanism of this phenomenon (41). MHC-based mating preferences were first identified in mice using laboratory tests (42), but the illustration that these preferences existed in naturalistic settings was first made in OPAs (43). This initial discovery spurred further research and now MHC-based mating preferences have been shown in over 20 species of vertebrates including amphibians, birds, fish, and reptiles (44-47). Likewise, the initial OPA discovery illustrates a fascinating discovery that led others to pursue its mechanistic underpinnings and it has now been shown that the peptides known to bind to MHC molecules also bind neuronal receptors in the vomeronasal organ (VNO) and the main olfactory epithelium (48, 49). Remarkably, the VNO sensory receptors bind 10-mer peptides with the same rules used by MHC molecules, where two of the peptides act as anchor positions for binding, while the other eight amino acids are free to vary without affecting binding. This amazing case of convergent evolution creates a seamless link between MHC-mediated immune recognition and MHC-mediated olfactory behaviors. Similarly, detecting disease phenotypes with OPAs offers a model system for discovery of mechanism that is impossible when the disease state remains cryptic.

References

1. Dimsdale JE. Psychological stress and cardiovascular disease. J Am Coll Cardiol. 2008 Apr 1;51(13):1237-46.

2. Lorvidhaya P, Stephen Huang SK. Sudden cardiac death in athletes. Cardiology. 2003;100(4):186-95.

3. Randolph C. An update on exercise-induced bronchoconstriction with and without asthma. Curr Allergy Asthma Rep. 2009 Nov;9(6):433-8.

4. Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol. 2000 Aug;32(1):56-67.

5. Gad SC. Animal models in toxicology. 2nd ed. Boca Raton: CRC/Taylor & Francis; 2007.

6. Drickamer LC. Semi-natural and enclosed groups of Macaca mulatta: a behavioral comparison. Am J Phys Anthropol. 1973 Sep;39(2):249-54.

7. Mossman CA, Drickamer LC. Odor preferences of female house mice (Mus domesticus) in seminatural enclosures. J Comp Psychol. 1996 Jun;110(2):131-8.

8. Sherborne AL, Thom MD, Paterson S, Jury F, Ollier WE, Stockley P, et al. The genetic basis of inbreeding avoidance in house mice. Curr Biol. 2007 Dec 4;17(23):2061-6.

9. Firman RC. Polyandrous females benefit by producing sons that achieve high reproductive success in a competitive environment. Proc Biol Sci. 2011 Sep 22;278(1719):2823-31.

10. Stolba A, Wood-Gush DGM. The behavior of pigs in a semi-natural environment. Animal Production. 1989 1989;48(2):419-26.

11. Davis JF, Krause EG, Melhorn SJ, Sakai RR, Benoit SC. Dominant rats are natural risk takers and display increased motivation for food reward. Neuroscience. 2009 Aug 4;162(1):23-30.

12. Duncan EA, Tamashiro KL, Nguyen MM, Gardner SR, Woods SC, Sakai RR. The impact of moderate daily alcohol consumption on aggression and the formation of dominance hierarchies in rats. Psychopharmacology (Berl). 2006 Nov;189(1):83-94.

13. Hurst JL. Behavioral variation in wild house mice Mus-domesticus rutty a quantitative assessment of female social organization. Animal Behaviour. 1987 1987;35(6):1846-57.

14. Wolff RJ. Mating behavior and female choice: their relation to social structure in wild caught House mice (Mus musculus) housed in a semi-natural environment. J Zool. 1985;207:43-51.

15. Bourin M, Hascoet M. The mouse light/dark box test. European Journal of Pharmacology. 2003 28 February,;463(1-3):55-65.

16. Sage RD. Wild mice. In: Foster HL, Small JD, Fox JG, editors. The mouse in biomedical research. N.Y.: Academic Press; 1981. p. 40-90.

17. Ilmonen P, Penn DJ, Damjanovich K, Clarke J, Lamborn D, Morrison L, et al. Experimental infection magnifies inbreeding depression in house mice. Journal of Evolutionary Biology. 2008;21:834-41.

18. Meagher S, Penn DJ, Potts WK. Male-male competition magnifies inbreeding depression in wild house mice. Proc Natl Acad Sci USA. 2000;97(7):3324-9.

19. Carroll LS, Meagher S, Morrison L, Penn DJ, Potts WK. Fitness effects of a selfish gene are revealed in an ecological context. Evolution. 2004;58:1318-28.

20. Latter BD. Mutant alleles of small effect are primarily responsible for the loss of fitness with slow inbreeding in Drosophila melanogaster. Genetics. 1998;148(3):1143-58.

21. Lynch CB. Inbreeding effects upon animals derived from a wild population of Mus musculus. Evolution. 1977;31:526-37.

22. Connor JL, Belucci MJ. Natural selection resisting inbreeding depression in captive wild house mice (Mus musculus). Evolution. 1979;33:929-40.

23. Bennett RI, Motulsky AG, Bittles A, Hudgins L, Uhrich S, Doyle DL, et al. Genetic counseling and screening of consanguineous couples and their offspring: recommendations of the national society of genetic counselors. Journal of Genetic Counseling. 2002;11(2):97-119.

24. Lewontin RC. The effect of differential viability on the population dynamics of *t* alleles in the house mouse. Evolution. 1968;22:262-73.

25. Addison J, McConnell EE. A review of carcinogenicity studies of asbestos and non-asbestos tremolite and other amphiboles. Regul Toxicol Pharmacol. 2007 Oct 11.

26. Rogan WJ, Chen A. Health risks and benefits of bis(4-chlorophenyl)-1,1,1-trichloroethane (DDT). Lancet. 2005 Aug 27-Sep 2;366(9487):763-73.

27. Heindel JJ. Role of exposure to environmental chemicals in the developmental basis of reproductive disease and dysfunction. Semin Reprod Med. 2006 Jul;24(3):168-77.

28. Phillips DH. Polycyclic aromatic hydrocarbons in the diet. Mutat Res.1999 Jul 15;443(1-2):139-47.

29. Jauniaux E, Burton GJ. Morphological and biological effects of maternal exposure to tobacco smoke on the feto-placental unit. Early Hum Dev. 2007 Nov;83(11):699-706.

30. Remig V, Franklin B, Margolis S, Kostas G, Nece T, Street JC. Trans fats in America: a review of their use, consumption, health implications, and regulation. J Am Diet Assoc. 2010 Apr;110(4):585-92.

31. Fung TT, Malik V, Rexrode KM, Manson JE, Willett WC, Hu FB. Sweetened beverage consumption and risk of coronary heart disease in women. Am J Clin Nutr. 2009 Apr;89(4):1037-42.

32. Ouyang X, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, et al. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. J Hepatol. 2008 Jun;48(6):993-9.

33. Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, et al. Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. Circulation. 2007 Jul 31;116(5):480-8.

34. Bray GA, Nielsen SJ, Popkin BM. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. Am J Clin Nutr. 2004 Apr;79(4):537-43.

35. Gross LS, Li L, Ford ES, Liu S. Increased consumption of refined carbohydrates and the epidemic of type 2 diabetes in the United States: an ecologic assessment. Am J Clin Nutr. 2004 May;79(5):774-9.

36. Stanhope KL. Role of fructose-containing sugars in the epidemics of obesity and metabolic syndrome. Annu Rev Med. 2012;63:329-43.

37. Marriott BP, Olsho L, Hadden L, Connor P. Intake of added sugars and selected nutrients in the United States, National Health and Nutrition Examination Survey (NHANES) 2003-2006. Crit Rev Food Sci Nutr. 2010 Mar;50(3):228-58.

38. Thresher JS, Podolin DA, Wei Y, Mazzeo RS, Pagliassotti MJ. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. Am J Physiol Regul Integr Comp Physiol. 2000 Oct;279(4):R1334-40.

39. Bocarsly ME, Powell ES, Avena NM, Hoebel BG. High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. Pharmacol Biochem Behav. 2010 Nov;97(1):101-6.

40. Greish K, Thiagarajan G, Herd H, Price R, Bauer H, Hubbard D, et al. Size and surface charge significantly influence the toxicity of silica and dendritic nanoparticles. Nanotoxicology. 2011 Jul 28.

41. Ruff JS, Nelson AC, Kubinak JL, Potts WK. MHC signaling during social communication. In: López-Larrea C, editor. Austin, Self and Nonself. Austin: Landes Bioscience; 2012. p. 290-313.

42. Yamazaki K, Boyse EA, Mike V, Thaler HT, Mathieson BJ, Abbott J, et al. Control of mating preferences in mice by genes in the major histocompatibility complex. J Exp Med. 1976;144:1324-35.

43. Potts WK, Manning CJ, Wakeland EK. Mating patterns in seminatural populations of mice influenced by MHC genotype. Nature. 1991;352:619-21.

44. Bos DH, Williams RN, Gopurenko D, Bulut Z, Dewoody J. Condition-dependent mate choice and a reproductive disadvantage for MHC-divergent male tiger salamanders. Molecular Ecology. 2009;18(15):3307-15.

45. Freeman-Gallant CR, Meguerdichian M, Wheelwright NT, Sollecito SV. Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. Molecular Ecology. 2003 Nov;12(11):3077-83.

46. Reusch TB, Haberli MA, Aeschlimann PB, Milinski M. Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. Nature. 2001 Nov 15;414(6861):300-2.

47. Olsson M, Madsen T, Nordby J, Wapstra E, Ujvari B, Wittsell H. Major histocompatibility complex and mate choice in sand lizards. Proceedings of the Royal Society of London Series B-Biological Sciences. 2003 Nov 7;270:S254-S6.

48. Leinders-Zufall T, Brennan P, Widmayer P, S PC, Maul-Pavicic A, Jager M, et al. MHC class I peptides as chemosensory signals in the vomeronasal organ. Science. 2004 Nov 5;306(5698):1033-7.

49. Spehr M, Kelliher KR, Li XH, Boehm T, Leinders-Zufall T, Zufall F. Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. J Neurosci. 2006 Feb 15;26(7):1961-70.

CHAPTER 2

HUMAN-RELEVANT INTAKE OF DIETARY FRUCTOSE DECREASES MOUSE COMPETITIVE ABILITY, SURVIVAL AND REPRODUCTION

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Summary paragraph

Health impacts of fructose intake at human-relevant concentrations have been difficult to study in rodent models, as unnaturally high doses have been required to demonstrate disease phenotypes. Fructose has increased in the American diet by 50% since the 1970s and over this same period the proportion of individuals suffering from metabolic diseases has dramatically increased[1]. Fructose consumption has been indicated as a factor in the development of cardiovascular disease, fatty liver, metabolic syndrome, obesity, and type-2 diabetes[2-6]. However, rodent studies concerning health impacts of fructose have exclusively focused on doses above 20% Kcal for liquid calories and 50% for dry, and therefore largely characterize effects that are outside of the range of typical

human exposure[7-10]. Here we report data produced by a novel methodology referred to as Organismal Performance Assays (OPAs), in which fructose-treated (12.5% Kcal) and control mice compete in seminatural enclosures for territories, resources, and mates. Within enclosures fructose-fed females experienced a two-fold increase in mortality while males fed fructose controlled 26% fewer territories and produced 25% less offspring. These findings represent the lowest observed adverse effect level (LOAEL) reported to date for fructose and highlight that fructose-induced physiological impairment can be substantial even when clinical endpoint measures are negative or inconclusive. These and other data suggest that OPAs are an innovative technique for detecting mammalian health decline and could have important utility in toxicity assessment of dietary components, environmental exposures, pharmaceuticals and other treatments.

Main body

Mechanisms for how fructose contributes to obesity, *de novo* lipogenesis, lipid deregulation and insulin resistance have been recently reviewed[11]. Support for these mechanisms is seen in rodent models where high-levels of fructose consumption has been shown to increase adiposity, levels of fasting cholesterol and triglycerides, impair glucose tolerance and promote inflammation[7-10]. However, rodent studies evaluating health impacts of fructose have exclusively focused on doses outside of the range of human exposure.

To sensitively assess whether the consumption of fructose decreases mouse health, as measured by survival, competitive ability and reproduction (common measures of evolutionary fitness), at human-relevant concentrations we utilized a novel technique, which we refer to as Organismal Performance Assays (OPAs). OPAs are sensitive

phenotyping approaches that use seminatural conditions to challenge the physiological performance of control and experimental animals in direct competition with each other. It is this competition that reveals performance differences between treatment and control individuals. The relative success of control and experimental animals can be compared for any measurable component of fitness. Though the OPA moniker has been recently derived, the technique has been used to detect mating preferences due to major histocompatibility genes and to quantify adverse consequences associated with cousin and sibling-level inbreeding as well as costs of bearing a selfish genetic element[12-15]. In all cases OPAs quantified substantial health impacts that had been missed by studies using standard laboratory methodologies.

Here we use OPAs to test if fructose exposure at a concentration of 12.5% Kcal, a level currently consumed by 13-25% of Americans, decreases mouse health[1,16]. Additionally, we monitor common metabolic endpoints between experimental and control animals to determine if established mechanisms correlate with whole organism phenotypes observed in OPAs.

Survival of female animals within OPA enclosures was impacted by diet, with fructose-fed females experiencing death rates 1.97 times higher than controls (Proportional Hazards (PH), $P = 0.048$); Fig. 2.1a). There was no difference in survival among replicate populations (PH, $P = 0.351$) nor did the impact of diet differ among replicate populations (PH, $P = 0.554$).

In regards to male survival, no relationship between diet and survival was detected (PH, $P = 0.777$); Fig. 2.1b). Survival did not differ among replicate populations

(PH, $P = 0.438$) nor did the impact of diet differ among replicate populations (PH, $P = 0.311$).

Male competitive ability was adversely affected by fructose feeding, with fructose-fed animals defending 25.9% fewer territories than control males throughout the study. At week three (model intercept) control males occupied 47.9% of territories and fructose-fed males only 35.5%. This difference was found to be significant (Generalized Linear Mixed Model (GLMM), $P = 0.036$). No effects of time or diet by time were detected on territorial acquisition indicating that the competitive advantage of control males was consistently maintained throughout the study. For a complete readout of all mixed model results see Table S2.1.

Female reproductive success was impacted by diet in two distinct and opposing ways (Fig. 2.2a). First, reproduction of control females at week eight (model intercept) was 23.81 ± 2.71 (M ± S.E.M.) offspring per population and for fructose-fed females it was 36.24 ± 3.11 offspring per population, this difference was significant (GLMM, $P < 0.0001$). Second, while the reproductive output of control females increased significantly over time at a rate of 1.02 ± 0.01 offspring per week (GLMM, $P = 0.042$), fructose-fed animals exhibited significantly reduced reproduction rate of -0.99 ± 0.00 offspring per week, the rates between fructose-fed and control females significantly differed (GLMM, $P < 0.0001$).

Male reproductive success was negatively impacted by diet, with fructose-fed males siring 25.3% fewer offspring per population than controls (Fig. 2.2b). Diet did not significantly affect the level of reproduction at week eight (model intercept) with control males producing 14.21 ± 1.88 (M ± S.E.M.) male offspring per population and fructose-

fed males producing 14.94 ± 1.99. However, there was a significant diet by time interaction causing fructose-fed males to sire 0.98 ± 0.05 fewer male offspring per week per population than controls (GLMM, $P = 0.035$). A marginally significant effect of time alone on male reproduction was also found (GLMM, $P = 0.088$).

Diet did not impact the mass of population founders at week zero (Fig. S2.1). Nor did the diets have differential impacts on mass over time or between the sexes.

Female glucose tolerance, as assessed by intraperitoneal glucose tolerance tests (IPGTT), was impacted by both diet and environment (Fig. 2.3a,c); fructose-fed females had decreased rates of glucose clearance overall (ANOVA, N = 39, $P = 0.037$) as did females in cages before OPA release compared to those inhabiting OPA enclosures (ANOVA, $P = 0.024$). No interaction between diet and environment was detected (ANOVA, $P = 0.182$). With posthoc tests, only the difference between dietary groups in cages prior to OPA entrance was found to be significant. In cages fructose-fed females had Area Under the Curve (AUC) values 1.42 times higher than controls (Fructose-fed 29,384 ± 2,597: Control 20,719 ± 1,692 mg/dL/120 minutes).

Male glucose clearance rates were not affected by diet (ANOVA, N = 25, $P = 0.519$), though, like females, there was a large effect of environment, with males in cages prior to OPA release having higher levels then postrelease (ANOVA, $P < 0.0001$; Fig. 2.3b,d). No interaction between diet and environment was detected (ANOVA, $P = 0.190$).

Fasting measures of plasma cholesterol, glucose, insulin, and triglycerides of both female and male animals prior to OPA entrance were not impacted by diet. All plasma measure data were also analyzed with the sexes combined revealing that total cholesterol

was 1.69 times higher in fructose-fed animals (t-test, t =2.271, df = 30, $P = 0.031$; Table S2.2).

Though nearly twice as many fructose-fed females died there was no clear pattern detected in regards to female reproductive success. Female reproduction was difficult to interpret as fructose-fed females had significantly higher reproduction early in the study as well as significantly lower reproduction as the study progressed. The decreased reproduction over time experienced by fructose-fed females was likely due to their significantly increased mortality. It is not surprising to see milder treatment-induced reproductive effects in females than males, as this has been seen in previous OPA studies[13-15].

Overall, fructose-fed males were outcompeted by control animals as measured by competitive ability and reproduction. Since death rates did not differ, it is likely that the lower reproduction of fructose males was due to their decreased ability to defend territories. The relationship between competitive ability and reproductive success is well established and has been seen before in OPAs[13-15].

Cholesterol was the only fasting plasma measure that may be predictive of the organismal impairment exhibited by fructose-fed animals in OPAs as no difference was seen in plasma glucose, insulin, or triglyceride concentrations. These data provide partial support that the fructose-fed animals may be suffering from increased levels of lipid deregulation prior to OPA entrance.

Impaired glucose clearance rates of fructose-fed females compared to controls prior to OPA entrance may reflect an as of yet to be identified physiological alteration that may underlie and be predictive of increased risk of death within OPA enclosures.

However, this impairment in glucose clearance disappears within two weeks of residing in OPAs, well before the majority of deaths have occurred. No similar observations were made in regards to male glucose clearance. The sex-specific nature of these findings is interesting and may be due to the intense metabolic demands experienced by females undergoing gestation and lactation. The finding that both dietary groups, as well as both sexes, markedly increased their rates of glucose clearance after entering OPAs is likely due to increased activity demanded by their new environment[17].

The above findings provide direct evidence of adverse health impacts due to fructose intake at 12.5% Kcal. The increased rates of mortality and decreased reproduction observed in this study now represent the LOAEL for dietary fructose. These adverse organismal-level findings are detectable while standard clinical measures are either unaltered, (mass, glucose, insulin, and triglycerides) or inconclusive (cholesterol and glucose tolerance), indicating that either our current mechanistic understanding of fructose induced toxicity is incomplete and/or that available clinical measures are not of sufficient sensitivity to reflect the physiological impairments leading to early death in females and drop in reproductive capacity in males.

We detected substantial adverse outcomes due to an added sugar exposure consisting of a 1:1 ratio of fructose and glucose amounting to 25% Kcal. Our results provide evidence that added sugar consumed at concentrations currently considered safe exerts dramatic adverse impacts on mammalian health[18,19]. Many researchers have already made calls for reevaluation of these safe levels of consumption[11]; whereas, others have advocated for more drastic regulatory measures to curtail sugar consumption[20].

Though OPAs detected profound differences in reproductive output and survival between fructose-fed and control animals the results from our studies are likely conservative. First, OPAs were terminated at 34 weeks because of the common, diet independent, high-rates of male attrition; it is likely that if the study continued for the entirety of the mouse lifespan that reproductive outputs between the treatments would continue to diverge. Second, at the start of OPA assessment all animals were put on the same fructose enriched diet, meaning that all of the adverse effects of the "fructose diet" are a consequence of exposure prior to OPA entrance. Third, our fructose diet was based on a modified chow and not a refined diet; meaning that our fructose-fed animals showed impairment despite having the remainder of their diet being highly nutritive with optimum mineral and vitamin composition.

Quantifying the ultimate negative impact on a mammal due to a treatment is a difficult undertaking and requires long-term studies that follow subjects, as they inhabit a relevant environment with associated stresses. Because of this, such studies have largely fallen under the purview of human epidemiology or clinical trials. By directly assessing the impacts of a treatment on the performance of house mice in OPAs we are capable of bridging the environmental relevance and longitudinal nature of human studies with the controllability and feasibility of animal models. These fructose data along with other similar successes using OPAs suggest that this and similar approaches will be an important tool in the detection and quantification of adversity caused by a wide array of treatments[13-15]. Since output measures (survival, competitive ability and reproduction) are similar across experiments, OPAs allow for the direct comparison of disparate treatments. For example our data indicate that this fructose diet is as detrimental to male reproduction

as cousin-level inbreeding (Fig. S2.2). Currently, there is a great need for sensitive toxicity assessment methods that work across a broad range of experimental manipulations. This need is particularly strong for both pharmaceutical science where 73% of drugs that pass preclinical trials fail due to safety concerns and for toxicology, where shockingly few compounds receive long-term testing[21,22].

Methods summary

Wild derived house mice were exposed under caged conditions to either a diet containing a 1:1 ratio of fructose and glucose monosaccharides amounting to 25% Kcal from added sugar (fructose diet), or to a control diet (free of added-sugar) from weaning through adulthood. Experimental and control animals (n=156) were then used to cofound six independent OPA populations (Fig. S2.3). Once in OPAs, all mice were fed the fructose diet. Populations were maintained for 32 weeks and differential performance between control and experimental founders was monitored for survival, competitive ability, and reproduction. Survival was ascertained by periodic checking for corpses, competitive ability through the use of passive integrated transponder (PIT) tags and PIT tag readers, and reproduction by genetically analyzing offspring produced within enclosures. Founder mass was assessed over the course of the study. In an additional population not used to assess any of the above endpoints, glucose tolerance was assessed in individuals before OPA entrance and again two weeks after release. Fasting cholesterol, glucose, insulin, and triglycerides, were also measured in a subset of animals at the end of the dietary exposure.

References

1 Vos, M. B., Kimmons, J. E., Gillespie, C., Welsh, J. & Blanck, H. M. Dietary fructose consumption among US children and adults: the Third National Health and Nutrition Examination Survey. *Medscape J Med* **10**, 160, (2008).

2 Fung, T. T. *et al.* Sweetened beverage consumption and risk of coronary heart disease in women. *Am J Clin Nutr* **89**, 1037-1042, (2009).

3 Ouyang, X. *et al.* Fructose consumption as a risk factor for non-alcoholic fatty liver disease. *J Hepatol* **48**, 993-999, (2008).

4 Dhingra, R. *et al.* Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. *Circulation* **116**, 480-488, (2007).

5 Bray, G. A., Nielsen, S. J. & Popkin, B. M. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. *Am J Clin Nutr* **79**, 537-543, (2004).

6 Gross, L. S., Li, L., Ford, E. S. & Liu, S. Increased consumption of refined carbohydrates and the epidemic of type 2 diabetes in the United States: an ecologic assessment. *Am J Clin Nutr* **79**, 774-779, (2004).

7 Jurgens, H. *et al.* Consuming fructose-sweetened beverages increases body adiposity in mice. *Obes Res* **13**, 1146-1156, (2005).

8 Kelley, G. L., Allan, G. & Azhar, S. High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. *Endocrinology* **145**, 548-555, (2004).

9 Thresher, J. S., Podolin, D. A., Wei, Y., Mazzeo, R. S. & Pagliassotti, M. J. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. *Am J Physiol Regul Integr Comp Physiol* **279**, R1334-1340, (2000).

10 Bergheim, I. *et al.* Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. *J Hepatol* **48**, 983-992, (2008).

11 Stanhope, K. L. Role of fructose-containing sugars in the epidemics of obesity and metabolic syndrome. *Annu Rev Med* **63**, 329-343, (2012).

12 Potts, W. K., Manning, C. J. & Wakeland, E. K. Mating patterns in seminatural populations of mice influenced by MHC genotype. *Nature* **352**, 619-621, (1991).

13 Ilmonen, P. *et al.* Experimental infection magnifies inbreeding depression in house mice. *Journal of Evolutionary Biology* **21**, 834-841, (2008).

14 Meagher, S., Penn, D. J. & Potts, W. K. Male-male competition magnifies inbreeding depression in wild house mice. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3324-3329., (2000).

15 Carroll, L. S., Meagher, S., Morrison, L., Penn, D. J. & Potts, W. K. Fitness effects of a selfish gene are revealed in an ecological context. *Evolution* **58**, 1318-1328, (2004).

16 Marriott, B. P., Olsho, L., Hadden, L. & Connor, P. Intake of added sugars and selected nutrients in the United States, National Health and Nutrition Examination Survey (NHANES) 2003-2006. *Crit Rev Food Sci Nutr* **50**, 228-258, (2010).

17 Suzuki, M., Shindo, D., Kimura, M. & Waki, H. Effects of exercise, diet, and their combination on metabolic-syndrome-related parameters in OLETF rats. *Int J Sport Nutr Exerc Metab* **21**, 222-232, (2011).

18 Naitonal Research Council. *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)*. (The National Academies Press, 2005).

19 Dietary Guidelines Advisory Committee. Report of the Dietary Guidelines Advisory Committee (DGAC) on the Dietary Guidelines for Americans, (2010).

21 Lipsky, M. S. & Sharp, L. K. From idea to market: the drug approval process. *J Am Board Fam Pract* **14**, 362-367, (2001).

22 Nel, A., Xia, T., Madler, L. & Li, N. Toxic potential of materials at the nanolevel. *Science* **311**, 622-627, (2006).

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Author Contributions

J.S.R, M.K.S., and W.K.P designed the experiment. J.S.R., A.K.S, S.A.H, M.M.S., B.L.S, and L.C.M. maintained OPA enclosures and collected the associated data. J.S.R. and A.K.S collected plasma. S.H.G. obtained plasma measures. J.S.R analyzed data and wrote the manuscript. All authors discussed results and commented on the manuscript.

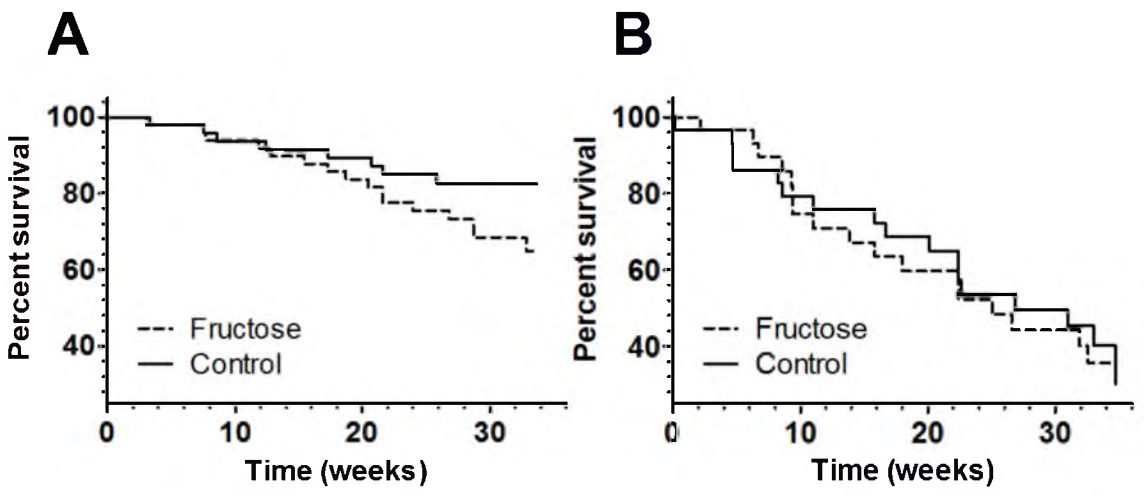

Figure 2.1. Survival of fructose-fed and control animals within OPA enclosures by sex. a, Fructose-fed females experienced a death rate twice that of control females (P = 0.048). **b,** This pattern was not not seen in males (P = 0.778).

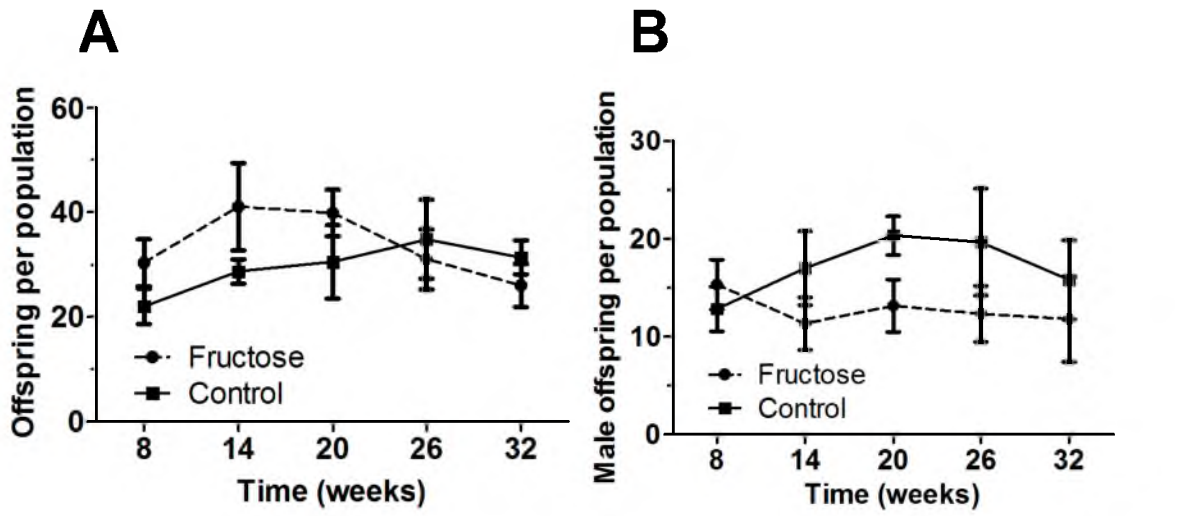

Figure 2.2. Reproduction of fructose-fed and control animals within OPA enclosures by sex. a, Fructose-fed females produced significantly more offspring early in the study ($P < 0.0001$), though this effect was negated due to a significant decrease in fructose-fed female reproduction over time ($P < 0.0001$). **b,** Male reproductive success was negatively impacted by diet, as fructose-fed males had a 25% reduction in reproductive output relative to controls ($P = 0.036$). Lines connect means and error bars represent standard error.

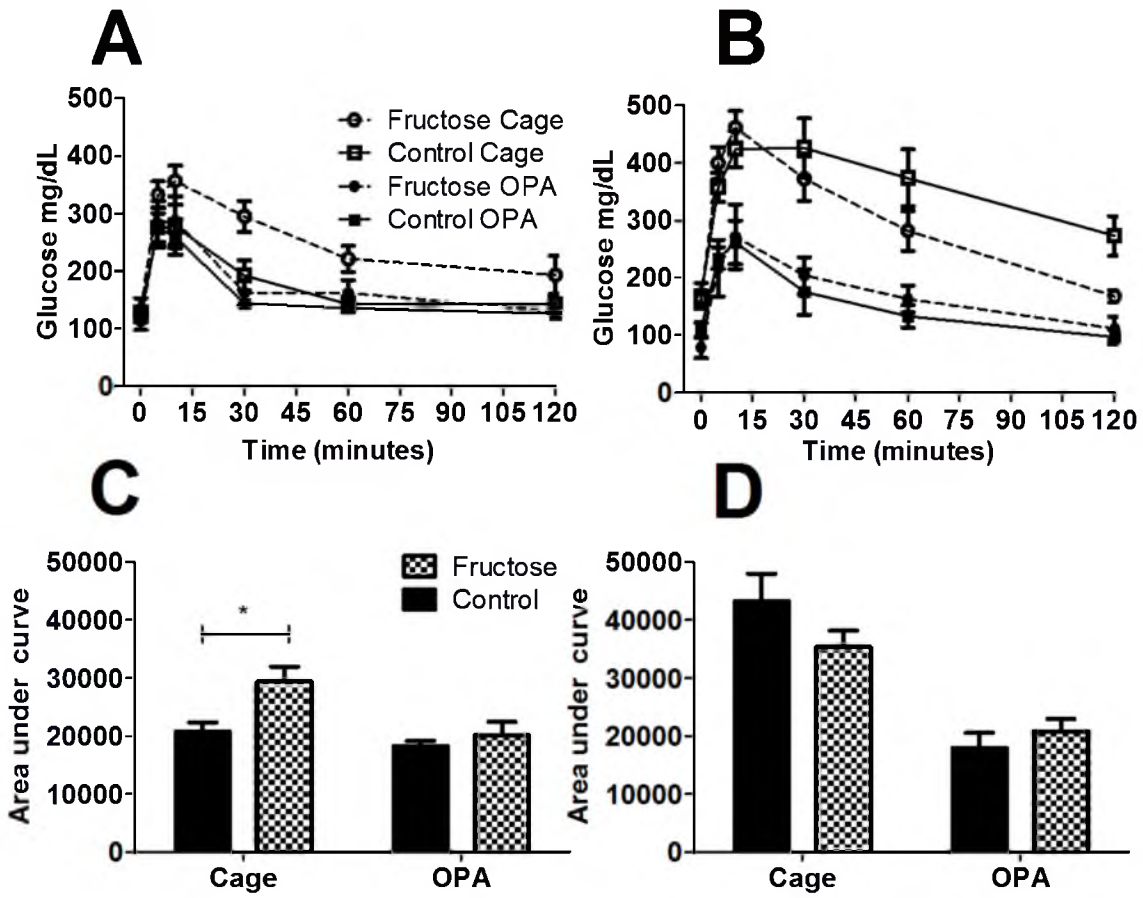

Figure 2.3. Glucose tolerance of female a,c, and male b,d, fructose-fed and control animals before and after OPA entrance as depicted by the glucose challenge time course plots a,b, and integrated area under the curve values c,d. Fructose-fed females had reduced glucose tolerance relative to controls ($P = 0.037$). Animals of both sexes and treatments had reduced glucose clearance prior to OPA release F:M ($P = 0.024$:$P < 0.0001$). Lines and bars represent means and error bars represent standard error. * Denotes significant ($P < 0.05$) posttest result.

Supplementary methods

Animals

Outbred, wild-derived house mice (*Mus musculus*) were used in this study, since many laboratory strains do not possess the functional behaviors required for OPA assessment[1]. Individuals in this study were from the 10th and 11th generation of the colony originally described by Meagher et al.[2]. Before animals were released into OPA enclosures they were housed according to standard protocols under a 12:12h light:dark cycle with food and water available *ad libitum*. All protocols were approved by and conducted under the animal care guidelines of the IACUC at the University of Utah.

Dietary exposure

Exposure to specified diets began at weaning and continued until animals were released into OPA enclosures approximately 26 weeks later. At the time of weaning a litter was split in half and assigned to either the treatment or control group. The Fructose diet (TD.05668) (Harlan Teklad, Madison, WI) contained 25% Kcal from a 1:1 mixture of fructose and glucose monosaccharides, and therefore has the same ratio of these monosaccharides as sucrose and approximately that of the 55:41 ratio found in the high fructose corn syrup (HFCS) used in soft drinks (or 42:53 ratio found in HFCS used in many food preparations). The control diet (TD.05669) (Harlan Teklad, Madison, WI) is identical except for the component coming from the fructose and glucose monosaccharides is replaced by cornstarch and a small amount of raw fiber used to offset mass differences (See Tables S2.3 and S2.4). Upon entrance into OPA enclosures all individuals consume the fructose diet.

OPA enclosures

OPA enclosures are indoors, measure about 5m by 7m ($35m^2$), and each pen is subdivided into six subsections by hardware cloth, which provides spatial complexity (Fig. S2.3). Each subsection has food and water that is associated with a set of nest boxes in either one of four "optimal" territories, which contain nest boxes in enclosed structures or two "suboptimal" territories with nest boxes in the open. Optimal nest boxes were made of covered plastic storage bins (75 liter) with 5cm diameter entryways and contained four standard mouse cages (also with 5cm entryways), bedding, and food. The suboptimal nest boxes made of plastic planter boxes (61cm long by 15cm wide by 19cm high) fitted with chicken-wire lids and 5cm circular entryways; food containers and one gallon poultry waterers were adjacent to these nest boxes and both provided *ad libitum* resource access. Together, the hardware fences and the two types of nest boxes created environmental complexity in which mice established nesting sites, territorial boundaries, and social hierarchies. OPA enclosures mimic habitat and social environment experienced by mice in nature and the population density is representative of measurements from wild populations[3].

To assess impacts of fructose consumption on survival, competitive ability and reproduction six OPA populations were founded by 22-28 individuals, 8-10 males and 14-18 females for a total of 156 individuals (58 male: 98 female). Equal numbers of fructose-fed and control animals were represented for each sex within all populations. No male individual was related at the cousin level or above to any other individual (male or female) within a given population. Relatedness between female founders was also avoided, though in several populations a single pair of sisters was included (a typical

condition in natural populations); when this was the case sister-pairs were balanced across diets. Mean age of individuals at the time of population founding was 29.83 ± 3.60 (M ± S.D.) weeks for males and 30.64 ± 3.60 weeks for females. To prevent incidental breeding before the establishment of male social territories, we released placeholder (nonexperimental) females with the experimental males at the onset of each population. After one week, the placeholder females were removed and the experimental females were released into the enclosures marking the start (week one) of the OPA portion of the study. Five of the six populations ran for 34 weeks, while the other replicate had to be terminated early at 26 weeks due to attrition. A seventh population was established under the same criteria above to collect blood samples from individuals under seminatural conditions and ran for only six weeks. This seventh population was not used to assess competitive ability, survival, or reproduction.

Male competitive ability

One week prior to entrance each founder was implanted with a unique passive integrated transponder (PIT) tag (TX1400ST, BioMark, Boise ID). A set of PIT antennae and readers (FS2001F-ISO, BioMark, Boise ID) were rotated through the six populations at regular intervals throughout the study and placed at each of the optimal and suboptimal feeders, and data were streamed to a computer equipped with data-logging software (Minimon, Culver City, CA). Male social dominance was assigned when a male had >75% of the PIT-tag reads at a single location over the course of a multi-day reader session, and territories were designated as controlled by a fructose or control-fed male based on the dietary exposure of the male controlling them. Female data were collected

but results are not reported here as not enough is known about female dominance behavior to use it as a measure of performance.

Survivorship

Survivorship of population founders was determined by periodic checks in each enclosure. Dead founders were identified by their PIT-tag ID or personalized ear punches and removed from enclosures. Date of death was estimated based on three factors: date of last check, the last date an animal was recorded at a feeding station, and the condition of the corpse.

Reproductive success

Samples to determine the reproductive success of founders were gathered during "pup sweeps" in which pups born during the previous cycle were removed from the population, sacrificed and tissue samples taken for genetic analysis. The first sweep occurred during week eight of the study and additional sweeps followed every six weeks. This schedule prevented offspring born in the enclosures from breeding. In five of the six populations five pup sweeps occurred while in the remaining replicate only four sweeps were conducted. A total of 1,894 individual samples were collected with an average of 315.67 ± 65.54 ($M \pm S.D.$) per population.

Population level reproductive success was determined for fructose and control groups as described previously[2]. Briefly, in each competition enclosure male and female founders of each treatment were categorized by a common allelic variant on the Y-chromosome and mitochondrial genome, respectively. Allelic assignments were reversed across populations to avoid possible confounding effects of allele types. We obtained

1836 (97% of total) mitochondrial and 870 Y- chromosome (92% of total assuming a 1:1sex ratio) genotypes.

Metabolic measures

In addition to OPA endpoints, traditional metabolic measures associated with fructose-induced disease were taken including body mass, glucose tolerance, plasma fasting cholesterol, glucose, insulin, and triglyceride concentrations. Body mass was assessed in the 156 animals that founded the six OPA enclosures described above at the time they were released into enclosures and at each of the pup sweeps, for a total of six time points. Glucose tolerance was assessed in a different set of individuals composed of 24 females (16 fructose-fed and 8 controls) and 16 males (8 fructose-fed and 8 controls) at two time points, prior to entrance into an OPA enclosure and again two weeks after release. Finally, fasting concentrations of plasma cholesterol, glucose, insulin, and triglycerides were assessed on a third set of 17 female (8 fructose-fed, 9 control) and 15 male (8 fructose-fed, 7 control) animals at the end of the dietary exposure period (i.e., the time point that the OPA founders were released into enclosures).

Intraperitoneal Glucose Tolerance Tests (IPGTTs) were conducted by giving an intraperitoneal injection of 1.5mg D-glucose/g body mass after an eight-hour fast. Blood was collected from the retro-orbital sinus prior to glucose injection and 5, 10, 30, 60 and 120 minutes postinjection. This fast duration and bleeding technique were selected because our wild-derived mice do not tolerate fasting or handling stress as well as laboratory strains. Blood samples were immediately centrifuged at 10,000g for 10 minutes after which 8-10μl of plasma was decanted and flash frozen. Samples were

shipped on dry ice to the CHORI and glucose concentrations were assessed by the hexokinase method[4] .

Plasma samples for fasting cholesterol, glucose, insulin, and triglycerides were collected and shipped in the same manner as those for IPGTT. Plasma glucose was measured as described above. To determine plasma total cholesterol and triglyceride concentrations, the Infinity Triglycerides or Cholesterol liquid stable reagent (Thermo Scientific), respectively, were employed. Briefly, plasma or standards were added in duplicate to a 96-well plate and the reagent was added and incubated at 37°C. Through a series of reactions, a colored dye was formed in proportion to the concentration of cholesterol or triglycerides and their levels were measured by the increase of absorbance at 500 nm. Plasma insulin was determined using a direct sandwich ELISA (Mercodia, Uppsala, Sweden). Briefly, plasma or standards were added to a detection antibody-coated 96-well plate. After incubation together with a peroxidase conjugated detection antibody, the substrate TMB was added and allowed to react and subsequently stopped with H_2SO_4. A colored product was formed in proportion to the concentration of insulin and its level was measured by the increase in absorbance at 450 nm. The homeostatic model assessment of insulin resistance (HOMA-IR) was calculated by first multiplying fasting insulin (mM) and glucose (mM) together, and then dividing by 22.5[5].

Statistical analysis

Survival. Survivorship of the 156 founders was analyzed by Cox proportional hazard models with male and female animals assessed separately due to vastly different mortality rates. Day one was defined as when animals entered OPA enclosures. A multivariate model was used to assess the impacts of diet, population, and diet by

population. Individuals that survived the duration of the trial or that were removed from the study were censored. In the male data set there were 34 events and 24 censorings while in the female data set there were 24 events and 74 censorings.

Male competitive ability. To assess the main effects of diet and time (and a time by diet interaction) on male competitive ability, we used a generalized linear mixed model (GLMM) to predict the probability of ownership. As a territory can only be defended or not, we used a binomial distribution with a logit link to assess the probability of ownership. The numbers of territories controlled within populations by each dietary treatment was assessed at multiple time points throughout the study for a total of 140 observations. The number of possible territories in each population is constant at six and territories were occupied (by a mouse of either diet) or unoccupied. Time, diet and their interaction were treated as fixed effects and population was modeled as a random effect with a random intercept calculated for each.

Reproduction. As reproduction data are discrete counts, for each sex we modeled offspring counts over time in a GLMM with a Poisson distribution and a logarithmic link. The model assessed the main effects of diet and time and the interaction on population-level reproduction across the six populations. Reproductive output of each dietary treatment was measured five times (except for one population that was only measured four times) at six-week intervals for a total of 58 observations. Time, diet and their interaction were modeled as fixed effects and population was modeled as a random effect with a random slope and intercept for females and only an intercept for males. The intercept was set at week eight as this was the first time point for which data were available and reproduction at week zero was biologically impossible. Male and female

reproduction data were analyzed separately as they were based on separate measurements, with male reproduction being in terms of number of male offspring and female reproduction in terms of total offspring.

Mass. A linear mixed-effects model (LMM) was used to assess the main effects of diet, sex and time, as well as their respective interactions on the mass of the 156 population founders. As mass data are continuous, a normal distribution was assumed. Diet, sex, time and their interaction were modeled as main fixed effects and individual and population were modeled as random effects with a random intercept. The intercept was set at week zero as this was the first time point for which data were available and made biological sense. Founders were weighed at week zero and surviving individuals were weighed across the five aforementioned pup sweeps for a total of 713 observations. Due to nested random effects within the model degrees of freedom are not readily calculable and therefore *P* values are not provided. The authors of the statistical package suggest that estimates with $|t| > 2$ are deemed significantly different from zero.

IPGTT. The area under the curve (AUC) was calculated for plasma glucose concentrations over time using the trapezoid rule. AUC values were calculated for all individuals prior to OPA entrance and two weeks postrelease. Male and female data were analyzed separately as sex has been shown to impact glucose tolerance[6]. AUC values were compared across time points and dietary treatments using two-way analysis of variance (ANOVA) with Bonferroni's posttests. Sample prior to OPA entrance was 23 females (15 fructose-fed and 8 controls) and 16 males (8 fructose-fed and 8 controls). While the sample two weeks postentrance had 16 females (8 fructose-fed and 8 controls) and 9 males (5 fructose-fed and 4 controls).

Fasting plasma cholesterol, glucose, insulin, triglycerides and HOMA-IR.

Plasma measures in 17 (8 fructose-fed, 9 control) female mice and 15 male mice (8 fructose-fed, 7 control) were compared between dietary treatments. Sexes were analyzed both separately and combined. Normality of each measurement was assessed with a Shapiro-Wilk normality test. Blood measurements that did not significantly differ from a normal distribution were assessed with t-tests, while those that differed significantly were analyzed with a Mann-Whitney U test. An F-test was used to tests for unequal variance of all blood measurements between dietary groups. All tests were two-tailed and all α values were 0.5. Summaries of normality, and F-test results may be found in Table S2.2.

All mixed-effects models were fit in R using the *glmer* or *lmer* functions of the lme4 library[7 8]. For all mixed-effects models several candidate models for the random effects terms were fit to the data including models estimating both intercept and/or slope for random effects. In all cases the model that explained at least some of the variance with random effects and had the lowest AIC score was selected. Neither the significance of any reported fixed effect nor the magnitude of the effect differed between models. Estimates (and significance) were consistent with those obtained when we ignored either the nested structure and repeated measurements of individuals within populations or repeated measurements of individuals or populations. Proportional hazard models were performed in JMP 9.0.3 (SAS institute Inc., Cary NC) and two-way ANOVAs, Mann-Whitney U tests, and t-test were performed in Prism 5.03 (Graphpad Software Inc, La Jolla CA). All α values are 0.05 and all tests were two-tailed.

Supplementary references

1 Manning, C. J., Potts, W. K., Wakeland, E. K. & Dewsbury, D. A. in *Chemical Signals in Vertebrates* Vol. VI (eds R.L. Doty & D. Müller-Schwarze) 229-235 (Plenum, 1992).

2 Meagher, S., Penn, D. J. & Potts, W. K. Male-male competition magnifies inbreeding depression in wild house mice. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3324-3329., (2000).

3 Sage, R. D. in *The Mouse in Biomedical Research* Vol. I (eds H.L. Foster, J.D. Smalll, & J.G. Fox) 40-90 (Academic Press, 1981).

4 Slein, M. W. *Methods of Enzymatic Analysis*. 117-123 (Academic Press, 1963).

5 Matthews, D. R. *et al.* Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**, 412-419, (1985).

6 Ayala, J. E. *et al.* Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech* **3**, 525-534, (2010).

7 R Development Core Team R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria, 2012).

8 Bates, D., Maechler, M., & Bolker, B. lme4: Linear mixed-effects models using S4 classes v. R package version 0.999375-42 (2011).

9 Ilmonen, P. *et al.* Experimental infection magnifies inbreeding depression in house mice. *Journal of Evolutionary Biology* **21**, 834-841, (2008).

10 Carroll, L. S., Meagher, S., Morrison, L., Penn, D. J. & Potts, W. K. Fitness effects of a selfish gene are revealed in an ecological context. *Evolution* **58**, 1318-1328, (2004).

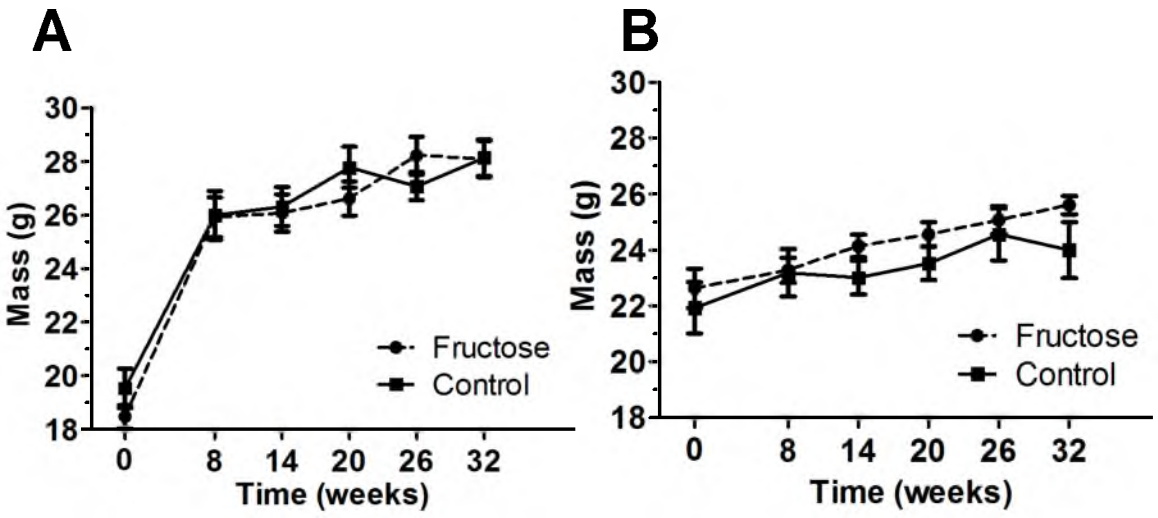

Figure S2.1. Body mass of OPA founders over time by sex. Diet did not impact the mass of either female **a,** or male **b,** animals (LMM; t = -0.87). Founders did significantly gain mass over time (LMM; t= 11.52) with males gaining at decreased rate compared to females (LMM; t= -6.01). The large change in female mass between weeks 0 and 8 is due to pregnancy. Lines connect means and error bars represent standard error.

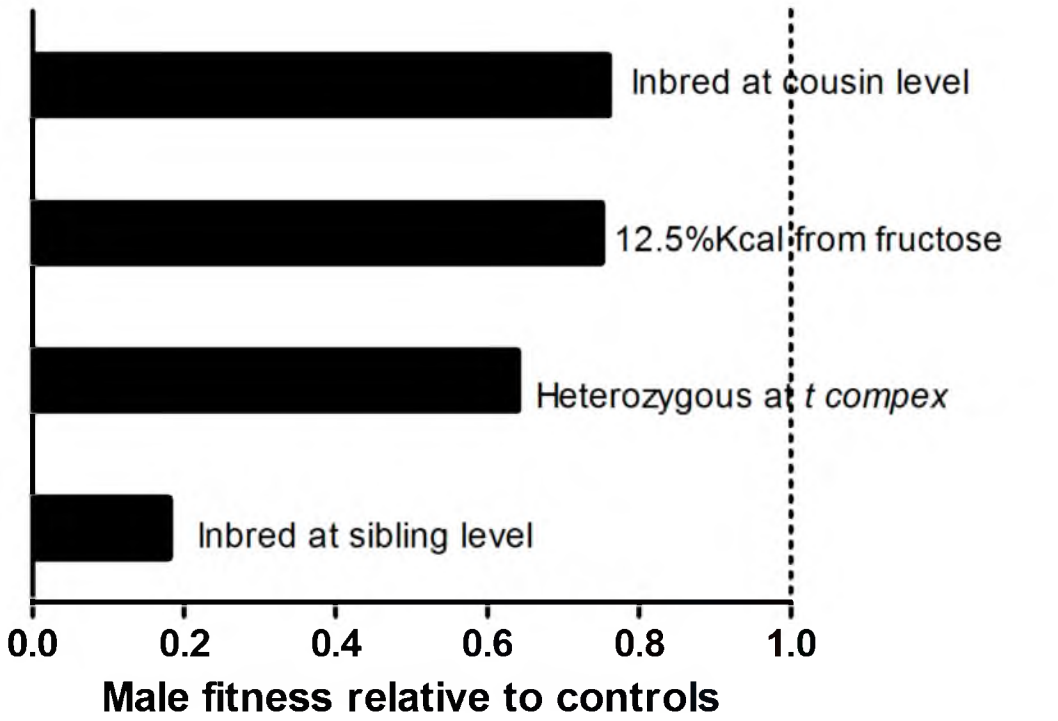

Figure S2.2. Relative male reproduction costs due to treatment from published OPA studies. Fructose-fed males experienced similar fitness declines to animals inbred at the cousin level, but not as severe as those bearing the selfish genetic element known as the *t complex* or inbred at the sibling level[2,9,10].

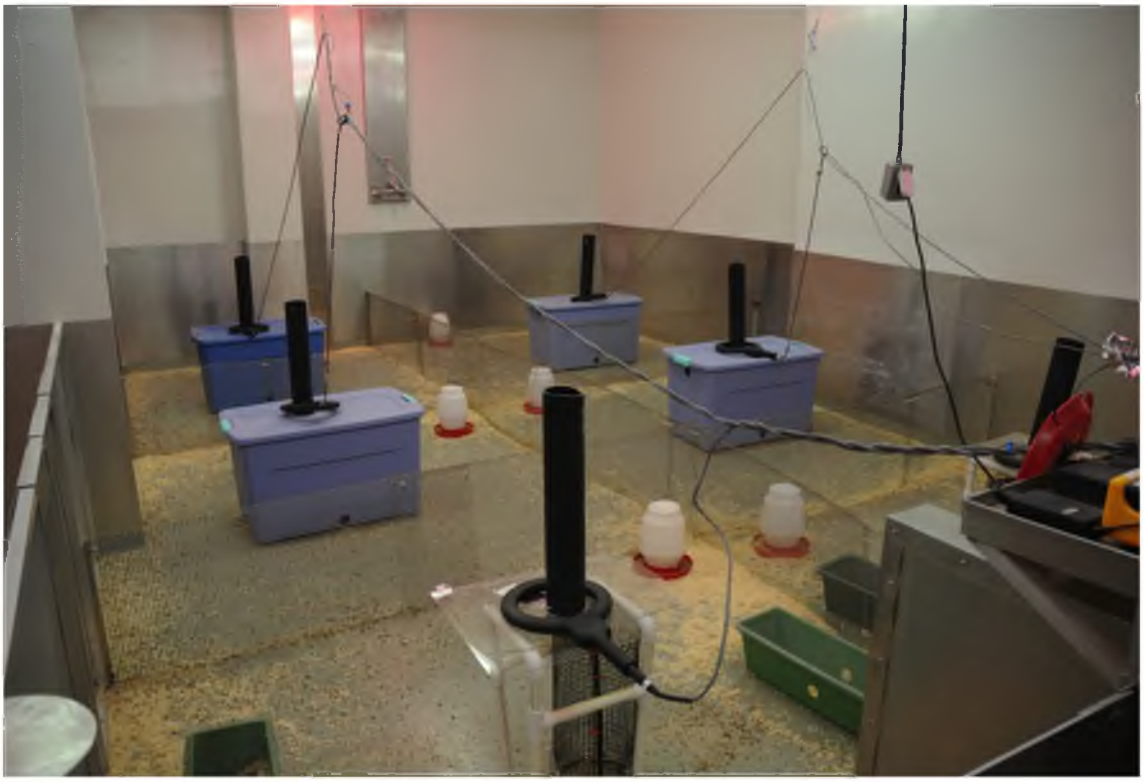

Figure S2.3. Photograph of OPA enclosure. Enclosures are approximately 35m^2, and are subdivided into six subsections by hardware cloth. Each subsection has food (black chimneys) and water (poultry waterers) that is associated with a set of nest boxes in either one of four "optimal" territories, which contain nest boxes in enclosed structures (storage tubs) or two "suboptimal" territories with nest boxes in the open (planter boxes with wire lids). PIT tag readers on the ledge of the enclosure are connected to antennae (black "tennis rackets"), which are placed over each feeding station. Photograph courtesy Ben Sutter.

Table S2.1. Mixed model results for competitive ability, reproduction and mass.

Male Competitive ability	GLMM with binomial distribution and logit link (Intercept at week 3)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.0023	0.0479		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	-0.0733	0.1666	-0.440	0.6600
Diet (Fructose)	-0.5054	0.2405	-2.102	0.0356*
Time	-0.0084	0.0114	-0.737	0.4610
Diet (Fructose)*Time	-0.0092	0.0169	-0.543	0.5869
Female Reproduction	GLMM with Poisson distribution and logarithmic link (Intercept at week 8)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.0458	0.2140		
Population (Slope)	0.0003	0.0183		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	3.1700	0.1078	29.398	<0.0001***
Diet (Fructose)	0.4204	0.0821	5.119	<0.0001***
Time	0.0174	0.0086	2.032	0.0422*
Diet (Fructose)*Time	-0.0236	0.0057	-4.157	<0.0001***
Male Reproduction	GLMM with Poisson distribution and logarithmic link (Intercept at week 8)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.0097	0.0984		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	2.6540	0.1245	21.311	<0.0001***
Diet (Fructose)	0.0503	0.1751	0.287	0.7738
Time	0.0092	0.0054	1.704	0.0884
Diet (Fructose)*Time	-0.0174	0.0082	-2.114	0.0345*
Mass	LMM (Intercept at week 0)			
Random effects	*Variance*	*Std. Deviation*		
Individual (Intercept)	5.58350	2.36294		
Population (Intercept)	0.48515	0.69653		
Fixed effects	*Estimate*	*Std. Error*	*t value*	*Significance (|t|>2)*
Intercept	21.6511	0.5929	36.52	Yes
Diet (Fructose)	-0.6141	0.7044	-0.87	No
Sex (Male)	0.5194	0.8099	0.64	No
Time	0.2394	0.0208	11.52	Yes
Diet (Fructose)*Sex (Male)	0.9643	1.0136	0.95	No
Diet (Fructose)*Time	0.0267	0.0268	1.00	No
Sex (Male)* Time	-0.1768	0.0294	-6.01	Yes

* Indicates a p value < 0.05., **< 0.01, ***< 0.001.

Table S2.2. Summary statistics of plasma measures

Plasma Measure	Sex	M ± S.D. (n) Fructose/Con.	Normally Distributed Fructose/Con.	Unequal Variance	*P*
Cholesterol mg/dL	Female	141.6 ± 40.58 (8) 116.5 ± 34.41 (9)	Yes/Yes	No	0.186
	Male	202.2 ± 23.95 (8) 146.9 ± 16.39 (7)	Yes/Yes	No	0.087
	Both	171.9 ± 15.59 (16) 129.8 ± 10.08 (16)	Yes/Yes	No	0.031 *
Glucose mg/dL	Female	102.7 ± 21.78 (8) 98.29 ± 21.98 (9)	Yes/No	No	1.000
	Male	136.4 ± 6.369 (8) 124.3 ± 4.269 (7)	Yes/Yes	No	0.148
	Both	119.5 ± 26.02 (16) 109.7 ± 22.04 (16)	Yes/No	No	0.396
Insulin ng/mL	Female	0.5747 ± 0.1271 (8) 0.3968 ± 0.05674 (9)	Yes/Yes	No	0.203
	Male	2.076 ± 2.290 (8) 1.283 ± 0.4124 (7)	No/Yes	Yes $F_{7,6}$=30.85 p=0.001***	0.694
	Both	1.325 ± 1.763 (16) 0.7846 ± 0.5382 (16)	No/Yes	Yes $F_{15,15}$=10.73 p<0.001***	0.356
Triglycerides	Female	44.18 ± 2.939 (8) 45.92 ± 2.172 (9)	Yes/Yes	No	0.634
	Male	47.84 ± 3.261 (8) 48.79 ± 2.565 (7)	Yes/Yes	No	0.826
	Both	46.01 ± 2.173 (16) 47.18 ± 1.643 (16)	Yes/Yes	No	0.671
HOMA-IR	Female	3.346 ± 2.547 (8) 2.036 ± 0.8298 (9)	No/Yes	Yes $F_{7,8}$=9.421 p=0.005**	0.236
	Male	16.46 ± 19.73 (8) 8.706 ± 3.221 (7)	No/No	Yes $F_{7,6}$=37.50 P<0.001***	0.867
	Both	9.904 ± 15.18 (16) 4.954 ± 4.024 (16)	No/No	Yes $F_{15,15}$=14.23 p< 0.001***	0.300

* Indicates a p value < 0.05., **< 0.01, ***< 0.001. Normality was assessed with a Shapiro-Wilk test and variance with an F-test.

Table S2.3. Formulation of fructose diet.

Fructose diet (TD.05668) 25% E from glucose + fructose					
Ingredient	**g/kg**	**% mass**	**Protein g/kg**	**CHO g/kg**	**Fat g/kg**
Wheat, Hard Ground	335.00	33.5	46.57	178.89	6.03
Dextrose, Monohydrate (Cerelose)	111.00	11.1	0	101.18	0
Fructose	101.00	10.1	0	101	0
Corn, Ground	95.00	9.5	7.695	65.74	3.04
Corn Gluten Meal 60	50.00	5	30.35	12.74	1.1
Soybean Meal, 48%	200.00	20	96.6	51	1.8
Dicalcium Phosphate, FG	16.00	1.6	0	0	0
Calcium Carbonate, FG	13.00	1.3	0	0	0
Sodium Chloride NaCl	5.00	0.5	0	0	0
Mineral Mix, TD.80318	1.50	0.15	0.0813	0.6946	0.03212
Vitamin Mix, TD.81125	3.00	0.3	0.0918	0.7844	0.03627
TBHQ (Antioxidant)	0.008	0.0008	0	0	0
Corn Oil	40.00	4	0	0	40
Cellulose (Fiber)	29.49	2.949	0	0	0
Totals (g/kg)	1000	100	181.38	512.02	52.04
Summary data					
	Total		**Protein**	**CHO**	**Fat**
Diet %	*100*		*18.14*	*51.20*	*5.20*
kcal/kg	*3241.96*		*725.53*	*2048.08*	*468.35*
kcal %	*100*		*22.38*	*63.17*	*14.45*

Table S2.4. Formulation of control diet.

Control diet (TD.05669)					
Ingredient	**g/kg**	**% mass**	**Protein g/kg**	**CHO g/kg**	**Fat g/kg**
Wheat, Hard Ground	335.00	33.5	46.57	178.89	6.03
Corn Starch	225.00	22.5	0.72	202.5	0.45
Corn, Ground	95.00	9.5	7.70	65.74	3.04
Corn Gluten Meal 60	50.00	5	30.35	12.74	1.1
Soybean Meal, 48%	200.00	20	96.6	51	1.8
Dicalcium Phosphate, FG	16.00	1.6	0	0	0
Calcium Carbonate, FG	13.00	1.3	0	0	0
Sodium Chloride NaCl	5.00	0.5	0	0	0
Mineral Mix, TD.80318	1.50	0.15	0.0813	0.6946	0.0321
Vitamin Mix, TD.81125	3.00	0.3	0.0918	0.7844	0.0363
TBHQ (Antioxidant)	0.008	0.0008	0	0	0
Corn Oil	40.00	4	0	0	40
Cellulose (Fiber)	16.49	1.649	0	0	0
Totals (g/kg)	1000.00	100.00	182.10	512.34	52.49
Summary data					
	Total		**Protein**	**CHO**	**Fat**
Diet %			*18.21*	*51.23*	*5.25*
kcal/kg	*3250.18*		*728.41*	*2049.38*	*472.40*
kcal %			*22.41*	*63.05*	*14.53*

CHAPTER 3

MODERATE LEVELS OF FRUCTOSE AND GLUCOSE MONOSACCHARIDES INCREASE DEATH RATES AND REDUCE FITNESS OF FEMALE MICE COMPARED TO THOSE FED SUCROSE

Abstract

Intake of added sugar has been shown to correlate with the prevalence of many human metabolic diseases and rodent models have characterized many aspects of the resulting disease phenotypes. However, very little work has been done to address if differential health impacts occur due to the consumption of one, or the other, of the two common types of added sugar, high fructose corn syrup (fructose and glucose monosaccharides) or table sugar (the disaccharide of sucrose, which is composed of fructose and glucose monosaccharides). To address this question directly, we fed mice either a diet containing an equal ratio of fructose and glucose monosaccharides or one with an isocaloric amount of sucrose. Exposure lasted from weaning through adulthood, and then animals of both treatments were released into seminatural enclosures where they competed for territories, resources and reproduction. Here, we report that female mice fed a diet containing an equal ratio of fructose and glucose monosaccharides experienced a mortality rate 1.87 times higher and produced 26.4% fewer offspring than females fed the sucrose diet. Interestingly, no differential performance was seen in male animals,

indicating a sex-specific outcome of exposure. This study provides the first experimental evidence that fructose and glucose monosaccharides can be more deleterious to mammalian health than the disaccharide sucrose.

Introduction

Added sugars are defined as sugars and syrups that are added to foods during processing or preparation (1). The two most common forms of added sugar in the American diet are table sugar (sucrose) and high fructose corn syrup (HFCS) (fructose and glucose monosaccharides), which make up 44% and 42% of consumption annually (2). HFCS comes in two main forms, one that is 42% and another that is 55% fructose, with the remainder being glucose. Due to both varieties of HFCS being widely used, the fructose to glucose ratio that is actually consumed is approximately 1:1 (3). Though consumption of HFCS is high in the American population, globally its total consumption is only 8% compared to sucrose (4).

Consumption of added sugar has been linked to numerous human disease states and the rapid increase in the prevalence of these diseases is becoming one of the most pressing health concerns world-wide as noncommunicable diseases now kill more people globally than infectious disease (5). Specifically, through epidemiological studies, added sugar consumption has been linked with cardiovascular disease, fatty liver disease, metabolic syndrome, obesity and type-2 diabetes (3, 6-9). Diseases that impact 36%, 11%, 24%, 36% & 11% of American adults respectively (10-14).

The fructose portion of added sugar is suspected to be the detrimental component and mechanisms for how it contributes to obesity; *de novo* lipogenesis, lipid deregulation and insulin resistance have been recently reviewed (15). Support for these mechanisms

can be seen in rodent models where high-levels of fructose consumption has been shown to increase adiposity, increase levels of fasting cholesterol and triglycerides and impair glucose tolerance (16-18). Additionally, fructose consumption has been shown to increase portal vein concentrations of bacterial endotoxin and therefore inflammation (19). This indicates that fructose, as opposed to other carbohydrates, has a deleterious impact on health. However, rodent studies concerning health impacts of fructose, with the exception of the data presented in Chapter 2, have exclusively focused on doses above 20% of total calories and often above 50%. Therefore, these studies largely characterize effects that are above the range of typical human exposure.

Few experimental studies have attempted to differentiate health consequences of consuming a mixture of fructose and glucose monosaccharides and the disaccharide sucrose. Numerous studies have compared diets containing high levels of fructose alone to those containing isocaloric sucrose and concluded that free fructose is more detrimental than sucrose and therefore that sources of free fructose such as HFCS are more deleterious to health (20). Unfortunately, these studies do not control for the total amount of fructose between treatments, and use a fructose diet that is likely to never be experienced in the real world, where fructose is always found with glucose (21). Of the studies that have directly compared fructose and glucose monosaccharides to sucrose, only two have found significant differences. Thresher et al. (2000) found that rats fed a mixture of fructose and glucose had decreased glucose infusion rates required to maintain euglycemia compared to those fed sucrose, and MyPhoung et al. (2011) found that people absorb more fructose when consuming beverages sweetened with a mixture of fructose and glucose than they do with sucrose sweetened beverages and therefore have elevated

biomarkers associated with increased fructose consumption (e.g., higher blood pressure) (3, 22). A third study concluded that a mixture of fructose and glucose caused increased mass gain in male rats compared to sucrose, though this claim may be overstepping the data as a direct statistical comparison between these dietary groups was not made (23).

To sensitively assess whether the consumption of fructose and glucose monosaccharides decreases mouse health relative to the consumption of sucrose we utilized a novel technique, which we refer to as Organismal Performance Assays (OPAs). OPAs are defined as sensitive phenotyping approaches that use seminatural conditions to challenge the physiological performance of differentially treated animals (i.e., treatment and control) in direct competition with each other. The relative success of individuals in each group can be compared for any fitness measures allowing detection and quantification of any reduced performance due to treatment. Though the OPA moniker has only recently been derived, the technique has been previously used to detect mating preferences due to major histocompatibility genes and quantify adverse consequences associated with cousin and sibling-level inbreeding as well as bearing the selfish genetic element known as the *t complex* (24-27). In all cases OPAs detected and quantified substantial health impacts that had been missed by previous studies that assessed animals with standard laboratory methodologies.

Here we use OPAs to specifically test if a 1:1 ratio of fructose and glucose monosaccharides (fructose/glucose) at a level of 25% kcal, a level currently consumed by 13% of Americans (28), decreases mouse health compared to consumption of an isocaloric sucrose diet. OPA endpoint measures include survival, reproductive success

and male competitive ability. Additionally, we monitor mass and glucose tolerance of animals to determine if these measures are predictive of OPA outcomes.

Methods

Animals

Outbred, wild-derived house mice (*Mus musculus*) were used in this study, as many laboratory strains do not possess the natural and functional behaviors required for OPA assessment (29). Individuals in this study were from the 10^{th} and 11^{th} generations of the colony originally described by Meagher et al. (26). Consanguinity was assessed during the 11^{th} generation and found to be comparable to wild populations (30). Before animals were released into OPA enclosures they were housed according to standard protocols under a 12:12h light:dark cycle with food and water available *ad libitum*. All protocols were approved by and conducted under the animal care guidelines of the IACUC at the University of Utah.

Dietary exposure

Exposure to specified diets began at weaning and continued until animals were released into OPA enclosures approximately 40 weeks later. At the time of weaning a litter was split in half and each portion ascribed to either the fructose/glucose or sucrose group. The fructose/glucose diet (TD.05668) (Harlan Teklad, Madison, WI) contained 25% kcal from a 1:1 mixture of fructose and glucose monosaccharides, and approximately models high fructose corn syrup (HFCS). The sucrose diet (TD.05667) (Harlan Teklad, Madison, WI) is identical except for the component coming from the fructose/glucose monosaccharides is replaced by sucrose and has slightly less raw fiber

added to offset mass differences. For an exact makeup of each diet see Tables 3.1 and 3.2. Upon entrance into OPA enclosures all individuals consume the glucose/fructose diet.

OPA enclosures

Indoor OPA enclosures measure 5m by 7m ($35m^2$), and each pen is subdivided into six subsections by hardware cloth, which provides spatial complexity. Each subsection has food and water that is associated with a set of nest boxes in either one of four "optimal" territories, which contain nest boxes in enclosed structures or two "suboptimal" territories with nest boxes in the open. Optimal nesting structures were made of covered, opaque plastic storage bins (75L) with 5cm diameter entryways and contained four standard mouse cages (also with 5cm entryways), bedding and food. The suboptimal nest box is made of plastic planter boxes (61cm long by 15cm wide by 19cm high) fitted with chicken-wire lids and 5cm circular entryways; food containers and one-gallon poultry waterers were adjacent to these nest boxes and both provided *ad libitum* resource access. Together, the hardware fences and the two types of nest boxes created environmental complexity in which mice established nesting sites, territorial boundaries, and social hierarchies. OPA enclosures mimic habitat and social environments experienced by mice in nature and the population density is representative of measurements from wild populations (31).

To assess impacts of the consumption of differential forms of fructose on survival, competitive ability and reproduction, six OPA populations were founded by 24-30 individuals, 8-10 males and 14-20 females for a total of 160 individuals (56 male:104 female). Equal numbers of fructose/glucose and sucrose-fed animals were represented in

each sex within all populations. No male individual was related at the cousin level or above to any other individual (male or female) within a given population. Relatedness between female founders was also avoided, though in several populations a single pair of sisters was included; when this was the case, sister-pairs were balanced across diets. Mean age of founders was 44.43 ± 5.69 (M ± S.D.) weeks for males and 44.28 ± 5.90 weeks for females. To prevent incidental breeding before the establishment of male social territories, we released placeholder (nonexperimental) females with the experimental males at the onset of each population to allow male territory formation prior to release of experimental females. After one week, the placeholder females were removed and the experimental females were released into the enclosures marking the start (week one) of the study. The six populations ran for 32 weeks.

Survivorship

Survivorship of population founders was determined by periodic checks in each enclosure. Dead founders were identified by their PIT-tag ID or unique ear punches and removed from enclosures. Date of death was estimated based on three factors: date of last check, the last date an animal was recorded at a feeding station, and the condition of the corpse.

Reproductive success

Samples to determine the reproductive success of founders were gathered during "pup sweeps" in which pups born during the previous cycle were removed from the population, sacrificed and had tissue samples taken for genetic analysis. The first sweep occurred during week eight of the study and additional sweeps followed every six weeks.

This schedule prevented offspring born in the enclosures from breeding. In all six of the populations five pup sweeps occurred. A total of 1,397 individual samples were collected with 235.83 ± 96.20 (M ± S.D.) per population.

Population level reproductive success was determined for fructose/glucose and sucrose groups as described previously (26). Briefly, in each competition enclosure male and female founders of each treatment were categorized by a common allelic variant on the Y-chromosome and mitochondrial genome, respectively. Allelic assignments were reversed across populations to avoid possible confounding effects of allele types. We obtained 1,336 mitochondrial (95.63% of total) and 667 Y- chromosome (99.85% of total assuming a 1:1sex ratio) genotypes.

Male competitive ability

One week prior to entrance, each founder was implanted with a unique passive integrated transponder (PIT) tag (TX1400ST, BioMark, Boise ID). Individuals were monitored until release and no redness, swelling, or noticeable infection around the injection site was detected. A set of PIT antennae and readers (FS2001F-ISO, BioMark, Boise ID) were rotated through the six populations throughout the study and placed at each of the optimal and suboptimal feeders, and data were streamed to a computer equipped with data-logging software (Minimon, Culver City, CA). Male social dominance was assigned when a male had >75% of the PIT-tag reads at a single location over the course of a multi-day reader session, and territories were designated as controlled by a fructose/glucose-fed or sucrose-fed male based on the dietary exposure of the male controlling them. Female data were collected, but results are not reported here as

not enough is known about female dominance behavior to use it as a measure of performance.

Body mass

Body mass was assessed in the 160 animals that founded the six OPA populations described above at the time they were released into populations and at each of the aforementioned pup sweeps, for a total of six time points.

Glucose clearance

Intraperitoneal Glucose Tolerance Tests (IPGTT) were conducted on eight female animals of each dietary treatment at the end of the exposure period by giving an intraperitoneal injection of 1.5mg D-glucose/g body mass after an eight hour fast. Only female animals were assessed as previous work on this population has shown that male clearance rates are not impacted by this level of added sugar consumption compared to a sugar-free control (Chapter 2). Blood was collected from the retro-orbital sinus prior to glucose injection and at 5, 10, 30, 60 and 120 minutes postinjection. Fast duration and bleeding technique were selected because our wild-derived mice do not tolerate fasting or handling stress as well as laboratory strains. Blood samples were immediately centrifuged at 10,000g for 10 minutes after which 8-10μl of plasma was decanted and flash frozen. Samples were shipped on dry ice to the Children's Hospital of Oakland Research Institute, and glucose levels were assessed by the hexokinase method (32). Briefly, plasma or glucose standard were added in duplicate to a 96-well plate and a glucose reagent (Thermo Scientific, Middletown, VA) was added and incubated at 37°C. Through

a series of reactions NADH was formed in proportion to the concentration of glucose and its level was measured by the increase of absorbance at 340 nm.

Statistical methods

Survival. Survivorship of the 160 founders was analyzed by Cox proportional hazard models with male and female animals assessed separately due to vastly different mortality rates. Day one was defined as when animals entered OPA enclosures and is different by a week for males and females (see above). A multivariate model was used to assess the impacts of diet, population and their interaction. Individuals that survived the duration of the trial or that were removed from the study were censored. In the male data set there were 32 events and 24 censorings, while in the female data set there were 40 events and 64 censorings.

Male competitive ability. To assess the main effects of diet and time (and a time by diet interaction) on male competitive ability, we used a generalized linear mixed model (GLMM) to predict the probability of ownership. As a territory can only be defended or not, we used a binomial distribution with a logit link to estimate probability of ownership (defense). The numbers of territories controlled within populations by each dietary treatment was assessed at multiple time points throughout the study for a total of 112 observations. The number of territories in each population is constant at six, and territories were occupied (by a mouse of either diet) or unoccupied. The intercept of the model was set at week zero when males were released into the enclosures. Week, time, diet and their interaction were treated as fixed effects and population was modeled as a random effect with a random intercept calculated for each.

Reproduction. As reproduction data are discrete counts, for each sex we modeled offspring counts over time in a GLMM with a Poisson distribution and a logarithmic link. The model assessed the main effects of diet and time and the interaction on population-level fitness across the six populations. Reproductive output of each dietary treatment was measured five times at six-week intervals for a total of 60 observations. Time, diet and their interaction were modeled as fixed effects and population was modeled as a random effect with both a random slope and intercept calculated. The intercept was set at week eight as this was the first time point for which data were available and reproduction at week zero was biologically impossible. Male and female reproduction data were analyzed separately as they were based on separate measurements, with male fitness measured in terms of number of male offspring and female fitness in terms of total offspring.

Mass. A linear mixed-effects model (LMM) was used to assess the main effects of diet, sex and time, as well as their respective interactions on the mass of the 160 population founders. As mass data are continuous, a normal distribution was assumed. Diet, sex, time and their interaction were modeled as main fixed effects and individual and population were modeled as random effects with a random intercept. The intercept was set at week zero as this was the first time point for which data were available and made biological sense. Founders were weighed at week zero and surviving individuals were weighed across the five aforementioned pup sweeps for a total of 706 observations.

IPGTT. The area under the curve (AUC) was calculated for plasma glucose levels over time using the trapezoid rule. AUC values were calculated for eight individuals of each treatment and comparisons were made between groups using a Mann Whitney U-

test as the distribution of the sucrose data set was found to vary significantly from that of a normal distribution using a Shaprio-Wilk normality test.

All mixed-effects models were fit in R (33) using the *glmer* or *lmer* functions of the lme4 library. For all mixed-effects models several candidate models for the random effects terms were fit to the data, including models estimating both intercept and/or slope for random effects. In all cases the model that explained at least some of the variance with random effects and had the lowest AIC score was selected. Estimates (and significance) were consistent with those obtained when we ignored either the nested structure and repeated measurements of individuals within populations or the repeated measurements of individuals or populations. Proportional hazard models were performed in JMP 9.0.3 (SAS institute Inc., Cary NC) and two-way ANOVAs, were performed in Prism 5.03 (Graphpad Software Inc, La Jolla CA). All α values are 0.05 and all tests were two-tailed.

Results

Survival of female animals within OPA enclosures was impacted by diet, with fructose/glucose-fed females experiencing death rates 1.8734 times higher than sucrose-fed females (Proportional Hazards; $\chi^2 = 6.3834$, $P = 0.0115$) (Figure 3.1a). There was no difference in survival among replicate populations (Proportional Hazards; $\chi^2 = 3.8825$, $P = 0.5665$) nor did the impact of diet differ between populations (Proportional Hazards; $\chi^2 = 8.1634$, $P = 0.1475$).

In regards to male survival, no relationship between diet and survival was detected (Proportional Hazards; $\chi^2 = 2.6602$, $P = 0.1029$) (Figure 3.1b). Overall survival did not differ among replicate populations (Proportional Hazards; $\chi^2 = 4.1569$, $P =$

0.5271); however, survival of the two treatments did significantly vary across populations (Proportional Hazards; $\chi^2 = 11.6310$, $P = 0.0402$).

Male competitive ability was not impacted by diet, with fructose/glucose animals controlling approximately the same number of territories as sucrose males at week zero (the intercept) (GLMM; Z = -1.078, $P = 0.2809$) (Figure 3.2). Fructose/glucose-fed males controlled 39.2% and sucrose-fed males 32.8% of territories throughout the study, leaving approximately 28% unoccupied at any time. No effect of time (GLMM; Z = -1.328, $P = 0.1840$) or diet by time (GLMM; Z = 0.322, $P = 0.7476$) was detected on territorial acquisition. For a complete readout of all GLMM (competitive ability and reproduction) and LMM (mass) results see Table 3.3.

Female reproductive success was impacted by diet with fructose/glucose-fed females producing 26.4% fewer offspring than sucrose-fed females (Figure 3.3a). At week eight (the model intercept) fructose/glucose females produced 17.07 ± 2.35 (M ± S.E.M.) offspring per population, while sucrose animals produced 23.97 ± 2.45 offspring per population, this difference was found to be significant (GLMM; Z = 3.479, $P = 0.0005$). As there was no significant effect of time (GLMM; Z = 0.218, $P = 0.8275$) or a time by diet interaction (GLMM; Z = -0.401, $P = 0.6883$), the deficiency in offspring production suffered by fructose/glucose females at the intercept persisted throughout the duration of the study. In total, fructose/glucose-fed females produced 96.50 ± 20.32 offspring per population and sucrose-fed females produced 131.17 ± 23.81 offspring per population.

No clear pattern emerged in regards to male reproductive success and treatment. At week eight (the model intercept) fructose glucose-fed males sired 16.25 ± 3.46 male

offspring per population, while sucrose-fed males sired 22.66 ± 3.19 (M ± S.E.M) male offspring per population, this difference was significant (GLMM; Z = 2.569, $P = 0.0102$). Conversely, sucrose fed-males suffered a significantly decreased rate (-1.06 ± 0.01 male offspring per week) of reproduction in regards to time, while fructose/glucose-fed males reproduced steadily (GLMM; Z =5.986, $P < 0.0001$). No overall impact of time on reproduction was observed (GLMM; Z = 1.503, $P = 0.1329$). In total, fructose/glucose-fed males produced 66.67 ± 30.90 male offspring per population and sucrose-fed males produced 47.50 ± 20.26 male offspring per population.

Diet did not impact the mass of population founders at the intercept (week zero) with sucrose-fed animals weighing 1.05 ± 0.71g (M ± S.E.M) less than fructose/glucose-fed animals (LMM; t = -1.47) (Figure 3.4). In addition, diet had no impact over time (LMM; t = 0.67) nor did it differentially impact the sexes (LMM; t = 0.48). Female founders significantly gained mass (0.22 ± 0.02g per week) (LMM; t = 9.78) while males largely maintained their entrance masses, the rate differences between males and females were significant (LMM; t = -8.90). However, at the intercept male founders weighed 1.93 ± 0.84g more than females. Due to nested random effects (individuals within populations) contained in the model, degrees of freedom are not readily calculable and therefore *P* values are not provided. The authors of the statistical package suggest that estimates with $|t| > 2$ are deemed significantly different from zero.

Female glucose tolerance, as assessed by intraperitoneal glucose tolerance tests (IPGTT), was not impacted by diet with fructose/glucose-fed females having an AUC score (mg/dL/120 minutes) of 28945 ± 2812 (M ± S.E.M) and sucrose-fed females

having an AUC of 31086 ± 7233 (Mann Whitney, N = 16, U = 25.00, $P = 0.5054$). For glucose tolerance curves of each treatment see Figure 3.5.

Discussion

Within OPA enclosures, female mice on the fructose/glucose diet were dramatically outperformed by sucrose-fed females, as demonstrated by a two-fold higher death rate and a 26% relative reduction in reproductive output. These are the first experimental data to show differential health impacts of consuming fructose/glucose *versus* sucrose. Interestingly, the reproductive disadvantage of fructose/glucose-fed females is present from the start of the study, indicating that even before the unequal mortality rates were manifest, reproduction differentials were present. The increased female death rate observed here (1.873) is remarkably similar to the one (1.966) we detected in a previous OPA competition between animals on the same fructose/glucose diet *versus* a starch-based control, indicating that perhaps the increased mortality in the previous study was due to free fructose and not total fructose as originally concluded. However, to assess this question, a direct OPA comparison between sucrose and starch control mice is needed.

Unlike females, males on the fructose/glucose diet were not outcompeted by their sucrose treated brethren. Fructose/glucose-fed males gained equivalent numbers of territories and experienced similar levels of mortality. Likewise, no overall pattern emerged in regards to reproduction with sucrose-fed male producing more offspring early on, while fructose/glucose-fed males sired more towards the end of the study. This, in conjuncture with our pervious study (Chapter 2), indicates that though male competitive ability and reproduction in OPAs is devastated by the consumption of 25% kcal from

added sugar, the form of that added sugar, as either fructose and glucose monosaccharides or the disaccharide sucrose, appears inconsequential; though this should be confirmed with direct competition experiments.

The sex-specific nature of our OPA findings is surprising, but not unprecedented. Twice our system has previously revealed a similar mortality pattern, the first being when females harboring the selfish genetic element known as the *t complex* experienced an increased risk of mortality while males with the *t complex* did not (27), and the second being that females fed the fructose/glucose diet in a previous study had increased mortality compared to controls while male animals showed no difference (Chapter 2). It is fallacious to assume that experimental treatments will impact the sexes in exactly the same way, as major differences in life histories of female and male mice are well established. For example, a female mouse when pregnant consumes 18-25% more calories than when she is not and therefore is likely to respond differentially to a nutritional treatment than a male (34). Even without considering pregnancy status, sex-specific differences are well established in metabolic processes such as the insulin response for both mice and humans (35, 36). As its importance is being realized, sex-specific reporting of results is becoming increasingly common in human studies and we believe that the data herein also reflect its importance in animal models (37).

We found no evidence of differential mass gain or glucose tolerance between animals fed the fructose/glucose and the sucrose diet. This finding is consistent with our previous work in which animals fed the same fructose/glucose diet did not differ in mass from those fed a starch-based control diet (Chapter 2), but it does stand in contrast to conclusions reached by Bocarsly et al. (2010) possibly due to rat/mouse differences (23).

Interestingly, our mass data supports that house mice are sexually dimorphic, as males were heavier than nonpregnant females. This observation is consistent with most data, but recent investigations have revealed that some populations and age ranges of wild house mice that are sexually monomorphic (38, 39). Glucose clearance rates did not differ between females raised on either diet, indicating that glucose tolerance was not predictive of the observed mortality and reproductive deficiency. Both the clearance rates of fructose/glucose and sucrose-fed females were similar to those of female mice fed the same fructose/glucose diet in Chapter 2. This observation is in line with data gathered by Thresher and coworkers (2000), that showed overall glucose clearance rates did not differ between these groups (3). The Thesher study did detect differences in the glucose infusion rate required to maintain euglycemia; however, we did not assess that aspect of glucose homeostasis.

The mechanistic cause of female mortality and reproductive impairment due to the consumption of the fructose/glucose diet, as opposed to sucrose, is not known. We directly tested for differences in glucose homeostasis and mass gain, two outcomes that previously had been reported as being differentially impacted by similar diets, but no differences emerged. We did not directly assess the rate of fructose uptake after consumption, a third metric that has been shown to differ between similar diets in humans, leaving the possibility that our fructose/glucose fed animals absorbed a higher amount of fructose each time they fed over the feeding trial. If not due to an increase in total fructose intake, then it seems likely that whatever mechanism is at play is taking place at or prior to the absorption of these sugars by enterocytes, as the bond connecting the monosaccharide components of sucrose is hydrolyzed at this time (40). Regardless of

what mechanism is contributing to the increased mortality and decreased reproduction of females on the fructose/glucose diet, the organismal-level phenotype characterized herein should greatly aid in its elucidation.

Though previous claims have been made that HFCS and sucrose are not equivalent, this study provides the first clear experimental evidence that the consumption of a 1:1 ratio of fructose and glucose monosaccharides can dramatically decrease mammalian health compared to the intake of an isocaloric amount of sucrose. Moreover, the fructose and glucose monosaccharide diet used in this study contains these added sugars at levels that are consumed by 13% of the American population, indicating that human health may also be at risk (28). Though many aspects of fructose toxicity have been well described, there is still much that we can learn from the mouse, especially when we take the crucial role of environment into account for elucidating and exacerbating disease phenotypes.

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References

1. National Research Council. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (acronutrients): Washington DC.: The National Academies Press; 2005.

2. Marriott BP, Cole N, Lee E. National estimates of dietary fructose intake increased from 1977 to 2004 in the United States. J Nutr. 2009 Jun;139(6):1228S-35S.

3. Bray GA, Nielsen SJ, Popkin BM. Consumption of high-fructose corn syrup in beverages may play a role in the epidemic of obesity. Am J Clin Nutr. 2004 Apr;79(4):537-43.

4. Fereday N, Forber G, Girardello S, Midgley C, Nutt T, Powell N, et al. HFCS industry annual review-a year of changing expectations. Oxford, UK: LMC International Ltd.; 2007.

5. United Nations General Assembly. Prevention and control of non-communicable diseases a Report of the Secretary-General. 2011.

6. Fung TT, Malik V, Rexrode KM, Manson JE, Willett WC, Hu FB. Sweetened beverage consumption and risk of coronary heart disease in women. Am J Clin Nutr. 2009 Apr;89(4):1037-42.

7. Ouyang X, Cirillo P, Sautin Y, McCall S, Bruchette JL, Diehl AM, et al. Fructose consumption as a risk factor for non-alcoholic fatty liver disease. J Hepatol. 2008 Jun;48(6):993-9.

8. Dhingra R, Sullivan L, Jacques PF, Wang TJ, Fox CS, Meigs JB, et al. Soft drink consumption and risk of developing cardiometabolic risk factors and the metabolic syndrome in middle-aged adults in the community. Circulation. 2007 Jul 31;116(5):480-8.

9. Gross LS, Li L, Ford ES, Liu S. Increased consumption of refined carbohydrates and the epidemic of type 2 diabetes in the United States: an ecologic assessment. Am J Clin Nutr. 2004 May;79(5):774-9.

10. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Executive summary: heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation. 2012 Jan 3;125(1):188-97.

11. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002 Apr 18;346(16):1221-31.

12. Ford ES, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. Jama. 2002 Jan 16;287(3):356-9.

13. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of obesity in the United States 2009–2010. National Center for Health Statistics. Hyattsville, MD.. 2012.

14. Center for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. In: Department of Health and Human Services Center for Disease Control and Prevention, editor. Atlanta, GA. 2011.

15. Stanhope KL. Role of fructose-containing sugars in the epidemics of obesity and metabolic syndrome. Annu Rev Med. 2012;63:329-43.

16. Jurgens H, Haass W, Castaneda TR, Schurmann A, Koebnick C, Dombrowski F, et al. Consuming fructose-sweetened beverages increases body adiposity in mice. Obes Res. 2005 Jul;13(7):1146-56.

17. Kelley GL, Allan G, Azhar S. High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. Endocrinology. 2004 Feb;145(2):548-55.

18. Thresher JS, Podolin DA, Wei Y, Mazzeo RS, Pagliassotti MJ. Comparison of the effects of sucrose and fructose on insulin action and glucose tolerance. Am J Physiol Regul Integr Comp Physiol. 2000 Oct;279(4):R1334-40.

19. Bergheim I, Weber S, Vos M, Kramer S, Volynets V, Kaserouni S, et al. Antibiotics protect against fructose-induced hepatic lipid accumulation in mice: role of endotoxin. J Hepatol. 2008 Jun;48(6):983-92.

20. White JS. Straight talk about high-fructose corn syrup: what it is and what it ain't. Am J Clin Nutr. 2008 Dec;88(6):1716S-21S.

21. White JS. Weak association between sweeteners or sweetened beverages and diabetes. J Nutr. 2008 Jan;138(1):138; author reply 9.

22. Le MT, Frye RF, Rivard CJ, Cheng J, McFann KK, Segal MS, et al. Effects of high-fructose corn syrup and sucrose on the pharmacokinetics of fructose and acute metabolic and hemodynamic responses in healthy subjects. Metabolism. 2011 Dec 5.

23. Bocarsly ME, Powell ES, Avena NM, Hoebel BG. High-fructose corn syrup causes characteristics of obesity in rats: increased body weight, body fat and triglyceride levels. Pharmacol Biochem Behav. 2010 Nov;97(1):101-6.

24. Potts WK, Manning CJ, Wakeland EK. Mating patterns in seminatural populations of mice influenced by MHC genotype. Nature. 1991;352:619-21.

25. Ilmonen P, Penn DJ, Damjanovich K, Clarke J, Lamborn D, Morrison L, et al. Experimental infection magnifies inbreeding depression in house mice. Journal of Evolutionary Biology. 2008;21:834-41.

26. Meagher S, Penn DJ, Potts WK. Male-male competition magnifies inbreeding depression in wild house mice. Proc Natl Acad Sci USA. 2000;97(7):3324-9.

27. Carroll LS, Meagher S, Morrison L, Penn DJ, Potts WK. Fitness effects of a selfish gene are revealed in an ecological context. Evolution. 2004;58:1318-28.

28. Marriott BP, Olsho L, Hadden L, Connor P. Intake of added sugars and selected nutrients in the United States, National Health and Nutrition Examination Survey (NHANES) 2003-2006. Crit Rev Food Sci Nutr. 2010 Mar;50(3):228-58.

29. Manning CJ, Potts WK, Wakeland EK, Dewsbury DA. What's wrong with MHC mate choice experiments? In: Doty RL, Müller-Schwarze D, editors. Chemical signals in vertebrates. New York, N.Y.: Plenum; 1992. p. 229-35.

30. Cunningham CB, Ruff JS, Chase K, Potts WK, Carrier DR. Competitive ability in male house mice (Mus musculus): Heritability, influences of body size and litter demographics. In review.

31. Sage RD. Wild mice. In: Foster HL, Smalll JD, Fox JG, editors. The mouse in biomedical research. New York, N.Y.: Academic Press; 1981. p. 40-90.

32. Slein MW. Methods of enzymatic analysis. Bergmeyer HU, editor. New York, NY: Academic Press; 1963.

33. R Development Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2012.

34. Gittleman JL, Thompson SD. Energy allocation in mammalian reproduction. American Zoologist. 1988 1988;28(3):863-76.

35. Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, et al. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. Dis Model Mech. 2010 Sep-Oct;3(9-10):525-34.

36. Mittendorfer B. Insulin resistance: sex matters. Curr Opin Clin Nutr Metab Care. 2005 Jul;8(4):367-72.

37. Institute of Medicine. Sex-specific reporting of scientific research: A workshop summary: The National Academies Press; 2012.

38. Dewsbury DA, Baumgerdner DJ, Evans RL, Webster DG. Sexual dimorphism for body mass in 13 taxa of muroid rodents under laboratory conditions. J Mammal. 1980 Feb;61(1):146-9.

39. Haisova-Slabova M, Munclinger P, Frynta D. Sexual size dimorphism in free-living populations of Mus musculus: Are male house mice bigger? Acta Zoologica Academiae Scientiarum Hungaricae. 2010;56(2):139-51.

40. Riby JE, Fujisawa T, Kretchmer N. Fructose absorption. Am J Clin Nutr. 1993 Nov;58(5 Suppl):748S-53S.

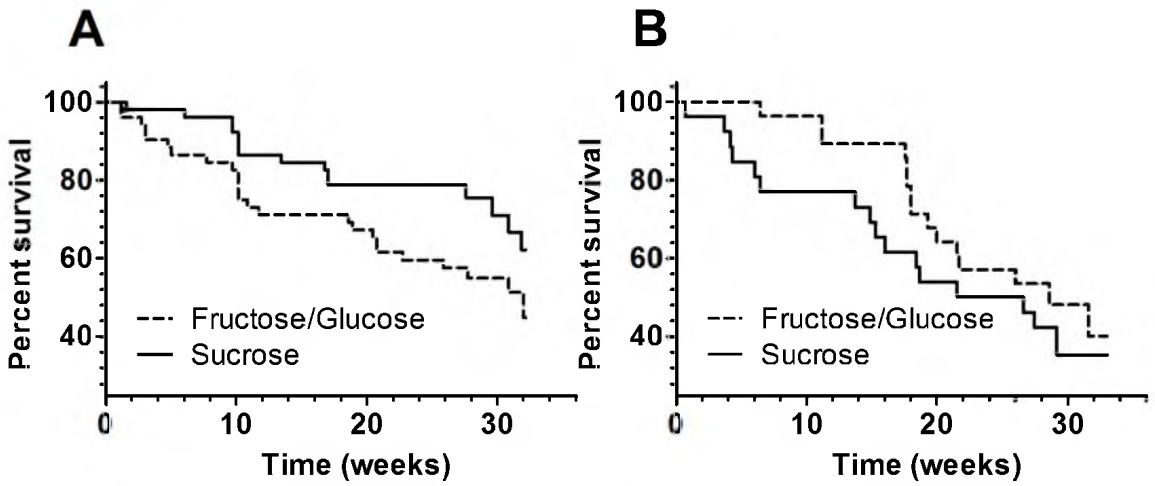

Figure 3.1. Survival of fructose/glucose and sucrose animals within OPA enclosures by sex. a, fructose/glucose-fed females experienced nearly twice the death rate over the course of the study compared to sucrose animals (Proportional Hazards; $\chi^2 = 6.3834$, $P = 0.0115$). **b,** No significant pattern was seen in males (Proportional Hazards; $\chi^2 = 2.6602$, $P = 0.1029$).

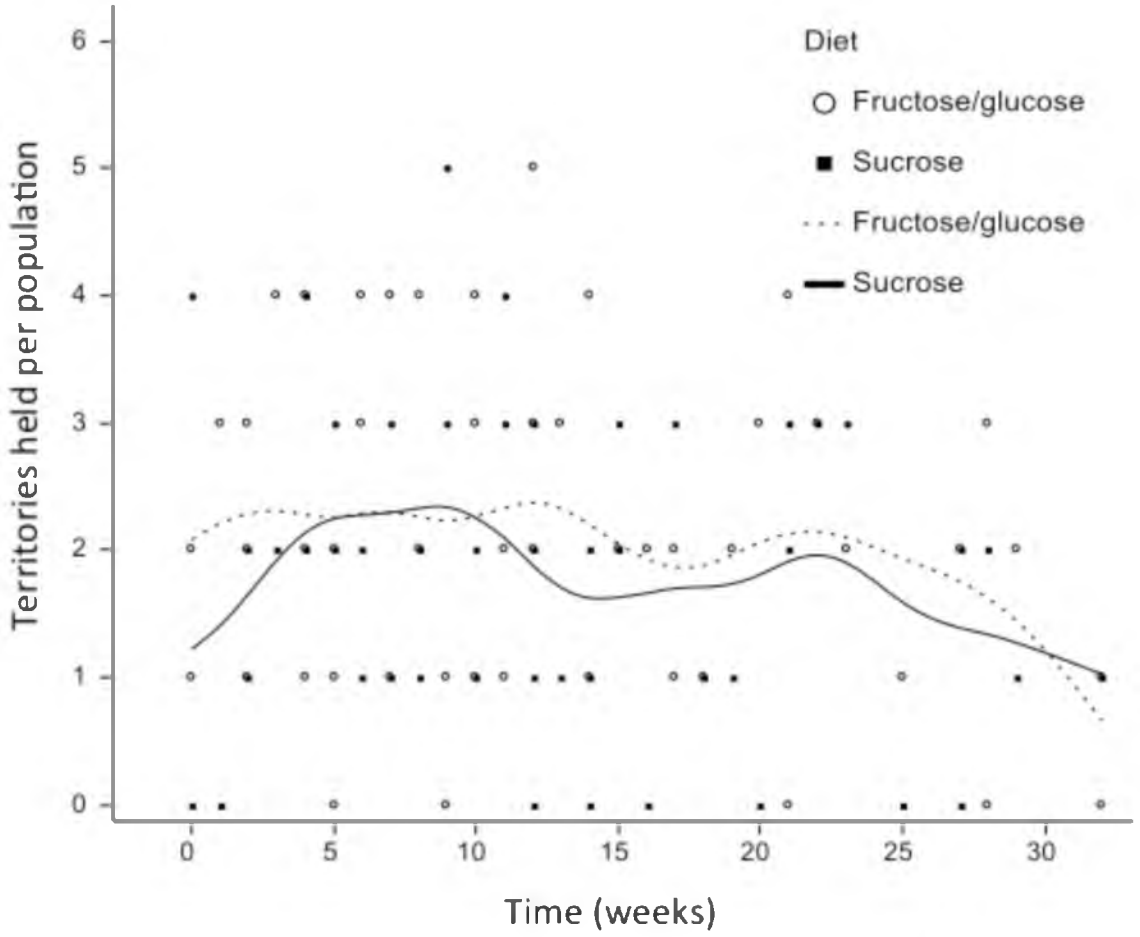

Figure 3.2. Male competitive ability over time. No difference in territorial acquisition between fructose/glucose and sucrose-fed males was detected (GLMM; Z = -1.078, *P* = 0.2809). Lines are smoothed representations of mean territoriality over time. Boxes/Circles represent the data points that inform the line.

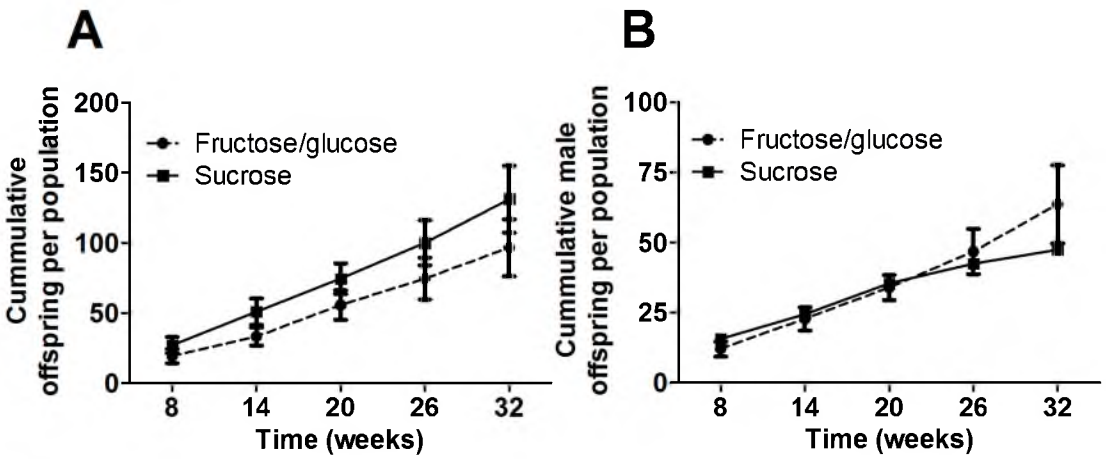

Figure 3.3. Cumulative reproductive success of OPA founders by sex. a, Fructose/glucose-fed females produced fewer offspring throughout the study than those fed sucrose (GLMM; $Z = 3.479$, $P = 0.0005$). **b,** Fructose/glucose-fed males produced significantly fewer offspring at the onset of the study (GLMM; $Z = 2.569$, $P = 0.0102$), but the advantage enjoyed by sucrose-fed males decayed over time (GLMM; $Z = 5.986$, $P < 0.0001$). Lines connects population means and errror bars represent standard error.

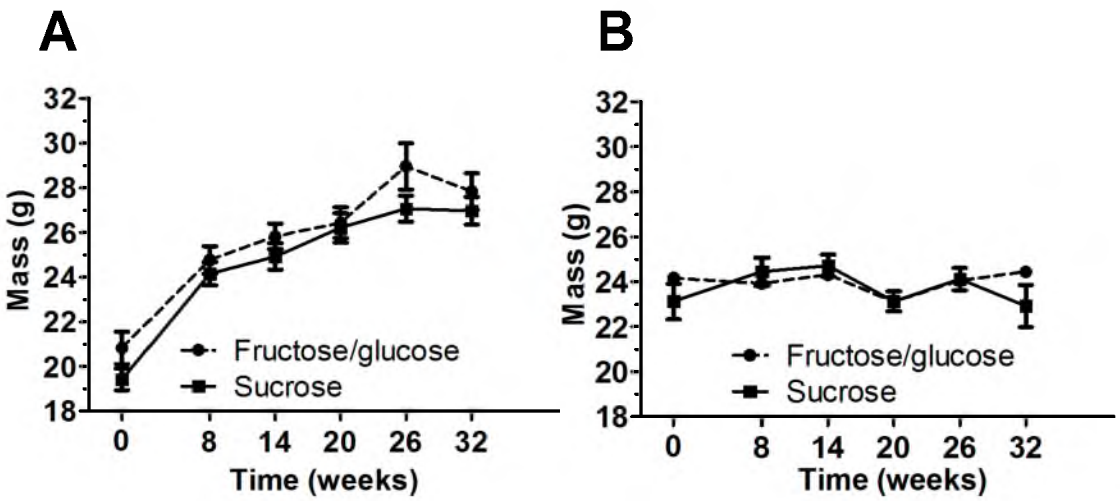

Figure 3.4. Body mass of fructose/glucose and sucrose animals within OPA enclosures by sex (a female, b male). No differences between treatment groups were observed (LMM; t = -1.47). Lines connect means of individuals assessed at OPA enterance and at the five pup sweeps, errror bars represent standard error.

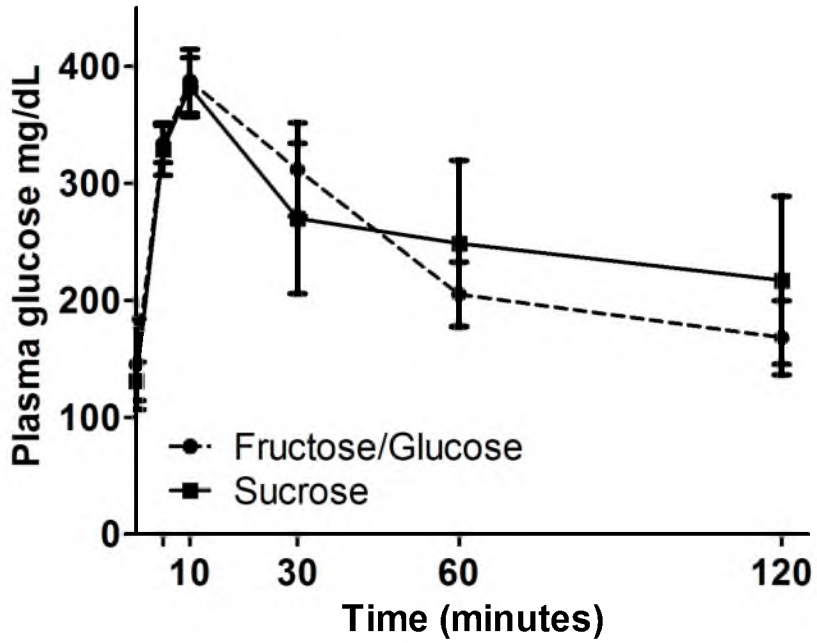

Figure 3.5. Glucose tolerance curves of fructose/glucose-fed and sucrose-fed females prior to release in OPA enclosures. No difference between treatment groups was observed (Mann Whitney, N = 16, U = 25.00, $P = 0.5054$). Lines connect means of individuals assessed at 0, 5, 10 , 30 , 60, and 120 minutes postglucose injection, errror bars represent standard error.

Table 3.1. Formulation of fructose/glucose diet.

Fructose/glucose diet (TD.05668) 25% kcal from glucose + fructose					
Ingredient	**g/kg**	**% mass**	**Protein g/kg**	**CHO g/kg**	**Fat g/kg**
Wheat, Hard Ground	335.00	33.5	46.57	178.89	6.03
Dextrose, Monohydrate (Cerelose)	111.00	11.1	0	101.18	0
Fructose	101.00	10.1	0	101	0
Corn, Ground	95.00	9.5	7.695	65.74	3.04
Corn Gluten Meal 60	50.00	5	30.35	12.74	1.1
Soybean Meal, 48%	200.00	20	96.6	51	1.8
Dicalcium Phosphate, FG	16.00	1.6	0	0	0
Calcium Carbonate, FG	13.00	1.3	0	0	0
Sodium Chloride NaCl	5.00	0.5	0	0	0
Mineral Mix, TD.80318	1.50	0.15	0.0813	0.6946	0.03212
Vitamin Mix, TD.81125	3.00	0.3	0.0918	0.7844	0.03627
TBHQ (Antioxidant)	0.008	0.0008	0	0	0
Corn Oil	40.00	4	0	0	40
Cellulose (Fiber)	29.49	2.949	0	0	0
Totals (g/kg)	1000	100	181.38	512.02	52.04
Summary data					
	Total		**Protein**	**CHO**	**Fat**
Diet %	*100*		*18.14*	*51.20*	*5.20*
kcal/kg	*3241.96*		*725.53*	*2048.08*	*468.35*
kcal %	*100*		*22.38*	*63.17*	*14.45*

Table 3.2. Formulation of sucrose diet.

Sucrose diet (TD.05667) 25% kcal sucrose					
Ingredient	**g/kg**	**% mass**	**Protein g/kg**	**CHO g/kg**	**Fat g/kg**
Wheat, Hard Ground	335.00	33.5	46.57	178.89	6.03
Sucrose	205.00	20.5	0	205	0
Corn, Ground	95.00	9.5	7.70	65.74	3.04
Corn Gluten Meal 60	50.00	5	30.35	12.74	1.1
Soybean Meal, 48%	200.00	20	96.6	51	1.8
Dicalcium Phosphate, FG	16.00	1.6	0	0	0
Calcium Carbonate, FG	13.00	1.3	0	0	0
Sodium Chloride NaCl	5.00	0.5	0	0	0
Mineral Mix, TD.80318	1.50	0.15	0.0813	0.6946	0.032
Vitamin Mix, TD.81125	3.00	0.3	0.0918	0.7844	0.0363
TBHQ (Antioxidant)	0.008	0.0008	0	0	0
Corn Oil	40.00	4	0	0	40
Cellulose (Fiber)	36.49	3.649	0	0	0
Totals (g/kg)	1000.00	100	181.38	514.84	52.04
Summary data					
	Total		**Protein**	**CHO**	**Fat**
Diet %			*18.14*	*51.48*	*5.20*
kcal/kg	*3253.25*		*725.53*	*2059.38*	*468.35*
kcal %	*100*		*22.30*	*63.30*	*14.40*

Table 3.3. Mixed model results for competitive ability, reproduction and mass.

Male Competitive Ability	GLMM with binomial distribution and logit link (Intercept at week 0)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.0518	0.2276		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	-0.4219	0.2277	-1.853	0.0639
Diet (Sucrose)	-0.2840	0.2916	-0.974	0.3302
Time	-0.0183	0.0138	-1.328	0.1840
Diet (Sucrose)*Time	0.0063	0.0194	0.322	0.7476
Female Reproduction	GLMM with Poisson distribution and logarithmic link (Intercept at week 8)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.0657	0.2563		
Population (Slope)	0.0005	0.0225		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	2.8375	0.1289	22.013	<0.0001***
Diet (Sucrose)	0.3392	0.0975	3.479	0.0005***
Time	0.0023	0.0105	0.218	0.8275
Diet (Sucrose)*Time	-0.0026	0.0064	-0.401	0.6883
Male Reproduction	GLMM with Poisson distribution and logarithmic link (Intercept at week 8)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.1678	0.4096		
Population (Slope)	0.0002	0.0145		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	2.2788	0.1932	11.797	<0.0001***
Diet (Sucrose)	0.3324	0.1294	2.569	0.0102*
Time	0.0130	0.0086	1.503	0.1329
Diet (Sucrose)*Time	-0.0566	0.0094	-5.986	<0.0001***
Mass	LMM (Intercept at week 0)			
Random effects	*Variance*	*Std. Deviation*		
Individual (Intercept)	9.1022	3.0170		
Individual (Slope)	0.0012	0.0343		
Population (Intercept)	0.1403	0.3745		
Fixed effects	*Estimate*	*Std. Error*	*t value*	*(|t|>2)*
Intercept	22.0016	0.5371	40.96	Yes
Diet (Sucrose)	-1.0471	0.7135	-1.47	No
Sex (Male)	1.9302	0.8395	2.30	Yes
Time	0.2155	0.0220	9.78	Yes
Diet (Sucrose)*Sex (Male)	0.5450	1.1288	0.48	No
Diet (Sucrose)*Time	0.0140	0.0210	0.67	No
Sex (Male)* Time	-0.2079	0.0234	-8.90	Yes

* Indicates a p value < 0.05., **< 0.01, ***< 0.001.

CHAPTER 4

ORGANISMAL PERFORMANCE ASSESSMENT OF AMINE-TERMINATED G7 PAMAM DENDRIMERS SUPPORTS THRESHOLD MODEL OF TOXICITY

Abstract

Engineered nanomaterials and their possible applications are rapidly multiplying and safety assessment of these compounds is failing to keep pace. One possible solution is determining if certain physicochemical properties influence toxicity in predictable ways. If so, toxicity could be predicted from structure and safety assessment could keep up with engineering advances. Here we use Organismal Performance Assays (OPAs) to determine if exposure to 3mg/kg amine-terminated G7 PAMAM dendrimers decreases mouse health and performance in a seminatural environment. This dose is the calculated maximum tolerated dose based on lethality due to blood coagulation at doses exceeding 10mg/kg. Within OPAs animals exposed to dendrimers did not experience increased mortality or decreased reproduction and exposed males exhibited significantly greater competitive ability and possessed 2.88 times more territories than control males. These OPA results suggest that an acute exposure of 3mg/kg amine-terminated G7 PAMAM dendrimers does not produce adverse mammalian health outcomes and that at levels

below those causing blood coagulation no other toxic mechanisms are associated with exposure, at least as detectable with OPAs.

Introduction

The volume of engineered nanomaterials and their possible applications have grown rapidly and though many benefits will come from this innovation, safety assessment is failing to keep pace. One possible solution is discovering if certain physicochemical properties (e.g., shape, size, surface area, and charge) influence toxicity is predictable ways. If toxicity could be predicted from structure, then safety assessment could keep up with advances in material science; but in order to insure accurate predictions we must have broad, sensitive and functional assays able to detect toxicity at the organismal level.

Size, surface properties and geometry of nanoparticles influence toxicity. Nanoparticles smaller than 20nm can transit through blood vessel walls or cross the blood brain barrier (1-3), while even smaller sized nanoparticles (~5nm) can be taken up by cells (4). In the cases described above, smaller nanoparticles are more toxic to cells than larger ones, but surface area is also highly important in regards to toxicity, with increasing surface area causing increased toxicity (5, 6). Modification of a nanoparticle's surface allows the binding of a variety of chemical, molecular and biological entities. Such manipulation can alter material properties concerning aggregation and solubility, and subsequently cellular transport and toxicity. For example, attachment of hydrophilic polymers, such as polyethylene glycol, to the surface of nanomaterials greatly increases solubility and the evasion of the reticuloendothelial system (4, 7). Particle charge also influences toxicity and the ability to traverse biological barriers (2, 8, 9), with cationic

particles being significantly more toxic than their anionic and neutral counterparts (10, 11).

Polyamidoamine (PAMAM) dendrimers are characterized by a unique tree-like branching architecture and a compact spherical shape in solution (12-14). The polydispersity values of PAMAM dendrimers range from ~ 1.000 – 1.005 verified by gel electrophoresis and mass spectrometry (15). Surface groups of PAMAM dendrimers can be cationic (e.g., amine terminated), anionic (e.g., carboxyl terminated) or neutral (e.g., hydroxyl). The high concentration of surface functionality and ability to manipulate this functionality through simple organic reactions has led to several applications of dendrimers in the coating of materials, electronics, and superconductors (16), as well as in drug delivery, bioseparation and diagnostic imaging (17, 18).The unique spherical shape, monodispersity and ability to incrementally increase size and number of surface functional groups make these structures suitable candidates for evaluating the influence of these physicochemical properties on their toxicity. Over the years Ghandehari and coworkers have demonstrated that cytotoxicity, cellular transport, and subcellular fate of PAMAM dendrimers can be modulated by controlling their size and surface properties (11, 19).

Here, we specifically assess the toxicity of amine-terminated generation seven (G7) PAMAM dendrimers at a dose of 3mg/kg body mass using organismal performance assays (OPAs). This dose is considered the current maximum tolerated dose (MTD) and is based on the observation that doses exceeding 10 mg/kg are lethal in mice, due to the aggregation of the particles and the resulting coagulation of blood (20). OPA assessment will determine if toxicity is present at the current MTD and, if present, will indicate that

the dendrimers may be promoting toxicity via an alternative mechanism as induced coagulation at this dose was not detected previously (20).

OPAs are defined as sensitive phenotyping approaches that use seminatural conditions to challenge the physiological performance of differentially treated animals (i.e., treatment and control) in direct competition with each other. The relative success of individuals in each group can be compared for any fitness measures allowing detection and quantification of any reduced performance due to treatment. Though the OPA moniker has only recently been derived, the technique has been previously used to detect mating preferences due to major histocompatibility genes, quantify adverse consequences associated with cousin and sibling-level inbreeding, and the selfish genetic element known as the *t complex* (21-24). In all cases OPAs detected and quantified substantial health impacts that had been missed by previous studies that assessed animals utilizing standard laboratory methodologies.

Methods

Animals

Outbred, wild-derived house mice (*Mus musculus*) were used in this study, as many laboratory strains do not possess the natural and functional behaviors required for OPA assessment (25). Individuals in this study were from the 12th generation of the colony originally described by Meagher et al. (23). Consanguinity was assessed during the 11th generation and found to be comparable to wild populations (26). Before animals were released into OPA enclosures they were housed according to standard protocols under a 12:12h light:dark cycle with food and water available *ad libitum*. All protocols

were approved by and conducted under the animal care guidelines of the IACUC at the University of Utah.

Dendrimer exposure

Exposure to dendrimers occurred intravenously through tail vein injection the day of OPA release. The dendrimers were constructed in the Ghandehari laboratory at the University of Utah and were suspended in sterile saline. Animals were weighed prior to injection and dosed at a level of 3mg/kg body mass. Control animals were handled in an identical matter except they were injected with only sterile saline.

OPA enclosures

Indoor OPA enclosures measure about 5m by 7m ($35m^2$), and each pen is subdivided into six subsections by hardware cloth, which provides spatial complexity. Each subsection has food and water that is associated with a set of nest boxes in either one of four "optimal" territories, which contain nest boxes in enclosed structures or two "suboptimal" territories with nest boxes in the open. Optimal nesting structures were made of covered, opaque plastic storage bins (75L) with 5cm diameter entryways and contained four standard mouse cages (also with 5cm entryways), bedding and food. The suboptimal nest boxes made of plastic planter boxes (61cm long by 15cm wide by 19cm high) fitted with chicken-wire lids and 5cm circular entryways; food containers and one gallon poultry waterers were adjacent to these nest boxes and both provided *ad libitum* resource access. Together, the hardware fences and the two types of nest boxes created environmental complexity in which mice established nesting sites, territorial boundaries, and social hierarchies. OPA enclosures mimic habitat and social environments

experienced by mice in nature, and the population density is representative of measurements from wild populations (27).

To assess impacts of amine-terminated G7 PAMAM dendrimer exposure at a dose of 3mg/kg on survival, competitive ability and reproduction, two OPA populations were founded by 10 males, and either 20 or 18 females, for a total of 58 individuals. Equal numbers of treatment and control animals were represented in each sex within both populations. No male individual was related at the cousin level or above to any other individual (male or female) within a given population. Relatedness between female founders was also avoided, though in each population a single pair of sisters was included, with one sister belonging to the treatment group and the other belonging to the control group. Mean age of founders was 18.44 ± 4.35 (M ± S.D.) weeks for males and 17.32 ± 4.55 weeks for females. Populations ran for a total of 18 weeks.

Survivorship

Survivorship of population founders was determined by periodic checks in each enclosure. Dead founders were identified by their PIT-tag ID or personalized ear punches and removed from enclosures. Date of death was estimated based on three factors: date of last check, the last date an animal was recorded at a feeding station and the condition of the corpse.

Reproductive success

Samples to determine the reproductive success of founders were gathered during "pup sweeps" in which pups born during the previous cycle were removed from the population, sacrificed and had tissue samples taken for genetic analysis. The first sweep

occurred during week eight and the second six weeks later. This schedule prevented offspring born in the enclosures from breeding. A total of 260 individual samples were collected between the populations.

Population level reproductive success was determined for treatment and control groups as described previously (23). Briefly, in each competition enclosure founders of each treatment group were chosen based on non-overlapping allelic assignments on the mitochondrial genome and Y-chromosome. Allelic assignments were reversed across populations to avoid possible confounding effects of allele types. In total we obtained 239 (91.9% of total) mitochondrial and 116 (89.2% of total assuming a 1:1 sex ratio) Y-chromosome genotypes.

Male competitive ability

One week prior to entrance each founder was implanted with a unique passive integrated transponder (PIT) tag (TX1400ST, BioMark, Boise ID). Individuals were monitored until release and no redness, swelling or noticeable infection around the injection site was detected. A set of PIT antennae and readers (FS2001F-ISO, BioMark, Boise ID) were rotated between the populations throughout the study, placed at each of the optimal and suboptimal feeders, and data were streamed to a computer equipped with data-logging software (Minimon, Culver City, CA). Male social dominance was assigned when a male had >75% of the PIT-tag reads at a single location over the course of a multi-day reader session, and territories were designated as controlled by a treatment or control male based on the exposure status of the male controlling them. Female data were collected, but results are not reported here as not enough is known about female dominance behavior to use it as a measure of performance.

Body mass

Body mass was assessed in the 58 animals that founded the OPA populations at both the time they were released into populations and at the first pup sweep occurring eight weeks later.

Statistical methods

Survival. Survivorship of the 58 founders was analyzed by Cox proportional hazard models with male and female animals assessed separately due to vastly different mortality rates. Day one was defined as when animals entered OPA enclosures and is different by a week for males and females (see above). In the male data set there were 6 events and 14 censorings while in the female data no mortality was observed so data were not analyzed.

Male competitive ability. To assess the main effects of diet and time (and a time by diet interaction) on male competitive ability, we used a generalized linear mixed model (GLMM) to predict the probability of ownership. As a territory can only be defended or not, we used a binomial distribution with a logit link to estimate probability of ownership (defense). The numbers of territories controlled within populations by the treatment or control group was assessed at multiple time points throughout the study for a total of 58 observations. The intercept was set at the time of OPA entrance, week zero. The number of territories in each population is constant at six and territories were occupied (by a mouse of either diet) or undefended. Time, diet and their interaction were treated as fixed effects and population was modeled as a random effect with a random intercept calculated for each.

Reproduction. Reproduction data were assessed using a Chi-square test with a Yates' correction on the total reproductive output of the populations combined. The number of expected events was determined by dividing the total number of offspring successfully genotyped by two. Male and female data were analyzed separately as they were ascertained with different genetic markers. Though the underlying assumption of each event (offspring) being independent may have been violated, this approach is used by other researchers using seminatural populations of mice to assess reproductive success (28).

Mass. A two-way analysis of variance (ANOVA) was used to assess the effects of exposure and time, as well as their interaction on the mass of the 58 population founders. Male and female masss were assessed separately as sexual mass dimorphism is present in our population (Chapter 3). All founders were weighed at the time of release and surviving animals were weighed again at the first pup sweep for a total of 103 observations.

Mixed-effects models were fit in R (29) using the *glmer* functions of the lme4 library. For mixed-effects models several candidate models for the random effects terms were fit to the data including models estimating both intercept and/or slope for random effects. In all cases the model that explained at least some of the variance with random effects and had the lowest AIC score was selected. Neither the significance of any reported fixed effect nor the magnitude of the effect differed between models. Estimates (and significance) were consistent with those obtained when we ignored either the nested structure and repeated measurements of individuals within populations or repeated measurements of individuals or populations. Proportional hazard models were performed

in JMP 9.0.3 (SAS institute Inc., Cary NC) and two-way ANOVAs and Yates' corrected Chi-square test with Prism 5.03 (Graphpad Software Inc, La Jolla CA). All α values are 0.05 and all tests were two-tailed.

Results

No relationship between male survival and dendrimer exposure was detected (Proportional Hazards; $\chi^2 = 1.8633$, $P = 0.1722$) (Fig. 4.1). Likewise, survival did not differ between the two populations (Proportional Hazards; $\chi^2 = 2.6845$, $P = 0.1013$) nor did the impact of diet differ between populations (Proportional Hazards; $\chi^2 = 2.5874$, $P = 0.1077$). Female survival was not assessed statistically as no females died within enclosures.

Male competitive ability was significantly impacted by treatment, with dendrimer exposed males controlling 2.88 times more territories than control males throughout the study (Fig. 4.2) At the intercept (week zero) control males possessed only 15.0% of territories, while treatment males controlled 43.0%, leaving 42% undefended; this difference was found to be significant (GLMM; $Z = 2.974$, $P = 0.0029$). Time positively impacted territorial acquisition (GLMM; $Z = 0.0876$, $P = 0.0052$) and there was no differential effect of time by treatment (GLMM; $Z = -1.375$, $P = 0.1690$). For a summary of GLMM results see Table 4.1.

Females exposed to dendrimers had 17.6% fewer offspring than controls, but this difference was not significant (Chi-square with Yates' correction; $\chi^2 = 0.927$, $P = 0.3356$). In total, treatment females had 108 offspring and control females had 131 offspring (Fig 4.3a). Likewise, no significant difference was seen in regards to male reproduction (Chi-

square with Yates' correction; $\chi^2 = 0.2764$, $P = 0.5991$). Treatment males sired 63 male offspring and control males had 53 male offspring (Fig 4.3b).

Dendrimer exposed female mice did not lose mass or gain less mass than control females (Fig. 4.4a). Mass at the time of injection and OPA entrance for treatment females were 15.16 ± 0.54g and control animals were 14.69 ± 0.52g; after the first sweep eight weeks later treatment females measured 26.03 ± 1.00g and controls 25.09 ± 0.65g. Overall no significant effect of dendrimer exposure on mass was detected (ANOVA; $F_{1,66} = 1.118$, $P = 0.2941$). The mass of all females did significantly increase from entrance to the first sweep (ANOVA; $F_{1,66} = 224.6$, $P < 0.0001$), though there was no interaction between exposure and time (ANOVA; $F_{1,66} = 0.1535$, $P = 0.6964$).

Like their female counterparts the mass of male mice was not impacted by exposure (Fig. 4.4b). Male masses at injection and OPA entrance were 18.46 ± 0.74g for treatment individuals and 19.17 ± 0.80g for controls; after the first sweep eight weeks later treatment males measured 23.81 ± 0.69g and controls 24.18 ± 0.97g. Overall no significant effect of dendrimer exposure on mass was detected (ANOVA; $F_{1,29} = 0.4195$, $P = 0.5223$). The mass of all males did significantly increase from entrance to the first sweep (ANOVA; $F_{1,29} = 38.80$, $P < 0.0001$), though there was no interaction between exposure and time (ANOVA; $F_{1,29} = 0.0424$, $P = 0.8383$).

Discussion

Exposure to 3/mg/kg of amine-terminated G7 PAMAM dendrimers did not negatively impact the mass, survival or reproductive output of mice in OPAs relative to controls. Furthermore, exposure may have enhanced male competitive ability. Though the scope of this OPA assessment was small compared to previous studies, two instead of

six replicate populations, the fact that all OPA metrics were negative is strong evidence that a single exposure to these dendrimers does not negatively impact the health and performance of house mice. In fact, the increased competitive ability of males that received the dendrimers is curious and warrants further study, though this effect may be significant in regards to these two OPA populations the extrapolation of this result to other populations would benefit from an increased sample size.

This OPA assessment represents the first case where a treatment assessed by this technique has not been found to reduce health and performance. In the three previously published studies and the two assessments of dietary fructose, at least one OPA endpoint measure (competitive ability, reproduction and survival) for one sex or the other has been adversely impacted by treatment (22-24)(Chapters 2 & 3). Though one can never prove a substance to be 100% safe we believe that powerful evidence is provided when OPAs fail to detect adversity, indicating that a substance is not likely to cause harm to mouse (mammalian) health.

As amine-terminated G7 PAMAM dendrimers have been shown to be lethal at acute doses exceeding 10 mg/kg, our observations that 3/mg/kg does not adversely impact health supports a threshold model of toxicity (20). Specifically, as the coagulation of the blood is the suspected toxic mechanism at lethal doses, it seems that if a given dose is not sufficient to initiate coagulation then no adverse response occurs, thus creating a binomial situation where the outcomes are either no harm or death. Though this may be the case for acute toxicity it is possible that chronic exposure or exposure at a critical time point during development may have different consequences; we therefore caution that our results are only to be interpreted in the context of a one-time exposure.

Fortunately, as a primary use for these dendrimers is as drug delivery compounds one-time injections may be a relevant form of exposure; however, to understand the outcomes to of chronic exposure that may impact material manufacturers and other groups more research is needed.

Safety assessment of engineered nanomaterials is a growing area of toxicology. As the number of engineered nanomaterials grows, and their applications multiply, many calls have been made for adequate safety assessment (30, 31). To keep up with the current pace of innovation many approaches have focused on high throughput *in vitro* techniques (32). However, we argue that *in vivo* testing must be used to "ground" *in vitro* results, and that the crucial role played by an organism's natural environment in the expression of disease phenotypes must not be ignored.

Acknowledgements

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References

1. Bogdanov AA, Jr., Chen JW, Kang HW, Weissleder R. Magnetic resonance signal amplification probes. Ernst Schering Res Found Workshop. 2005(49):147-57.

2. Kreuter J. Influence of the surface properties on nanoparticle-mediated transport of drugs to the brain. J Nanosci Nanotechnol. 2004 May;4(5):484-8.

3. Vinogradov SV, Batrakova EV, Kabanov AV. Nanogels for oligonucleotide delivery to the brain. Bioconjug Chem. 2004 Jan-Feb;15(1):50-60.

4. Hardman R. A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors. Environ Health Perspect. 2006 Feb;114(2):165-72.

5. Oberdorster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, et al. Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol. 2005 Oct 6;2:8.

6. Brown DM, Wilson MR, MacNee W, Stone V, Donaldson K. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol Appl Pharmacol. 2001 Sep 15;175(3):191-9.

7. Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res. 2003 Nov;42(6):463-78.

8. Fenart L, Casanova A, Dehouck B, Duhem C, Slupek S, Cecchelli R, et al. Evaluation of effect of charge and lipid coating on ability of 60-nm nanoparticles to cross an in vitro model of the blood-brain barrier. J Pharmacol Exp Ther. 1999 Dec;291(3):1017-22.

9. Lockman PR, Koziara JM, Mumper RJ, Allen DD. Nanoparticle surface charges alter blood-brain barrier integrity and permeability. J Drug Target. 2004;12(9-10):635-41.

10. Kitchens KM, El-Sayed ME, Ghandehari H. Transepithelial and endothelial transport of poly (amidoamine) dendrimers. Adv Drug Deliv Rev. 2005 Dec 14;57(15):2163-76.

11. Kitchens KM, Kolhatkar RB, Swaan PW, Eddington ND, Ghandehari H. Transport of poly(amidoamine) dendrimers across Caco-2 cell monolayers: Influence of size, charge and fluorescent labeling. Pharm Res. 2006 Dec;23(12):2818-26.

12. D'Emanuele A, Attwood D. Dendrimer-drug interactions. Adv Drug Deliv Rev. 2005 Dec 14;57(15):2147-62.

13. Tomalia DA, Naylor AM, Goddard WA. Starburst dendrimers: molecular-level control of size, shape, surface chemistry, topology and flexibility from atoms to macroscopic matter. Angew Chem Int Ed Engl. 1990;29:138-75.

14. Tomalia DA. Starburst™/cascade dendrimer: fundamental building blocks for a new nanoscopic chemistry set. Aldrichim Acta. 1993;26:91-101.

15. Esfand R, Tomalia DA. Poly(amidoamine) (PAMAM) dendrimers: from biomimicry to drug delivery and biomedical applications. Drug Discov Today. 2001 Apr 1;6(8):427-36.

16. Service RF. Dendrimers: Dream molecules approach real applications. Science. 1995 Jan 27;267(5197):458-9.

17. Svenson S, Tomalia DA. Dendrimers in biomedical applications--reflections on the field. Adv Drug Deliv Rev. 2005 Dec 14;57(15):2106-29.

18. Wilbur DS, Pathare PM, Hamlin DK, Buhler KR, Vessella RL. Biotin reagents for antibody pretargeting. 3. Synthesis, radioiodination, and evaluation of biotinylated starburst dendrimers. Bioconjug Chem. 1998 Nov-Dec;9(6):813-25.

19. Sadekar S, Ghandehari H. Transepithelial transport and toxicity of PAMAM dendrimers: Implications for oral drug delivery. Adv Drug Deliv Rev. 2012 May 1;64(6):571-88.

20. Greish K, Thiagarajan G, Herd H, Price R, Bauer H, Hubbard D, et al. Size and surface charge significantly influence the toxicity of silica and dendritic nanoparticles. Nanotoxicology. 2011 Jul 28.

21. Potts WK, Manning CJ, Wakeland EK. Mating patterns in seminatural populations of mice influenced by MHC genotype. Nature. 1991;352:619-21.

22. Ilmonen P, Penn DJ, Damjanovich K, Clarke J, Lamborn D, Morrison L, et al. Experimental infection magnifies inbreeding depression in house mice. Journal of Evolutionary Biology. 2008;21:834-41.

23. Meagher S, Penn DJ, Potts WK. Male-male competition magnifies inbreeding depression in wild house mice. Proc Natl Acad Sci USA. 2000;97(7):3324-9.

24. Carroll LS, Meagher S, Morrison L, Penn DJ, Potts WK. Fitness effects of a selfish gene are revealed in an ecological context. Evolution. 2004;58:1318-28.

25. Manning CJ, Potts WK, Wakeland EK, Dewsbury DA. What's wrong with MHC mate choice experiments? In: Doty RL, Müller-Schwarze D, editors. Chemical Signals in Vertebrates. New York, N.Y.: Plenum; 1992. p. 229-35.

26. Cunningham CB, Ruff JS, Chase K, Potts WK, Carrier DR. Competitive ability in male house mice (Mus musculus): Heritability, influences of body size and litter demographics. In review.

27. Sage RD. Wild mice. In: Foster HL, Smalll JD, Fox JG, editors. The mouse in biomedical research. New York, N.Y.: Academic Press; 1981. p. 40-90.

28. Firman RC. Polyandrous females benefit by producing sons that achieve high reproductive success in a competitive environment. Proc Biol Sci. 2011 Sep 22;278(1719):2823-31.

29. R Core Development Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2012.

30. Colvin VL. The potential environmental impact of engineered nanomaterials. Nat Biotechnol. 2003 Oct;21(10):1166-70.

31. Nel A, Xia T, Madler L, Li N. Toxic potential of materials at the nanolevel. Science. 2006 Feb 3;311(5761):622-7.

32. Damoiseaux R, George S, Li M, Pokhrel S, Ji Z, France B, et al. No time to lose--high throughput screening to assess nanomaterial safety. Nanoscale. 2011 Apr;3(4):1345-60.

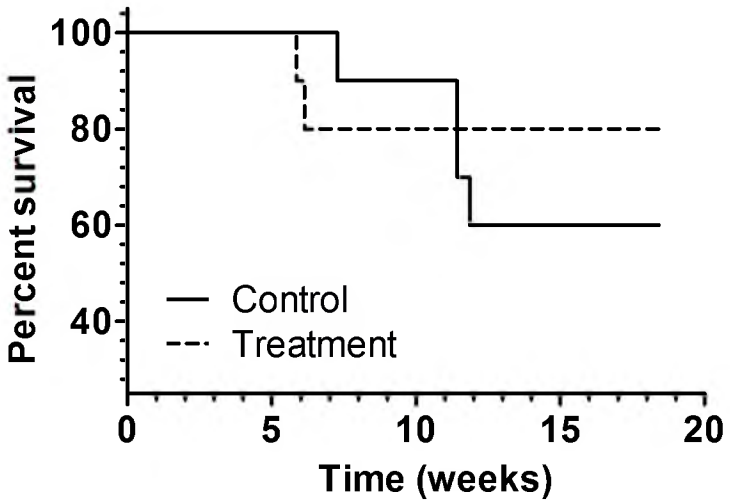

Figure 4.1. Survival of treatment and control males. Dendrimer exposure did not impact survival of male animals (Proportional Hazards; $\chi^2 = 1.8633$, $P = 0.1722$). Female data are not shown, as there was no female mortality.

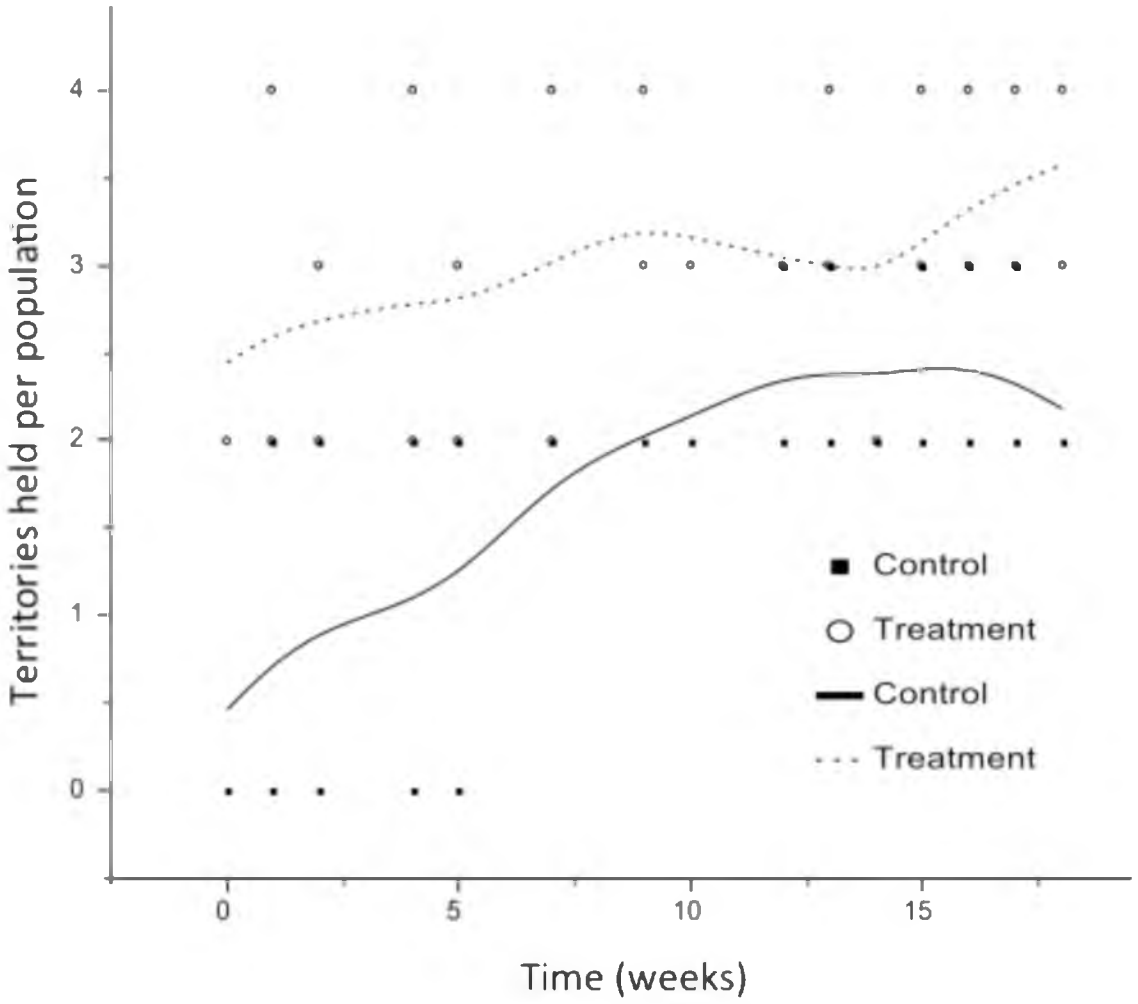

Figure 4.2. Male competitive ability over time. Dendrimer treated males controlled 2.88 times more territories throughout the study (GLMM; Z = 2.974, $P = 0.0029$). Lines are smoothed representations of mean territoriality over time. Boxes/Circles represent the data points that inform the line.

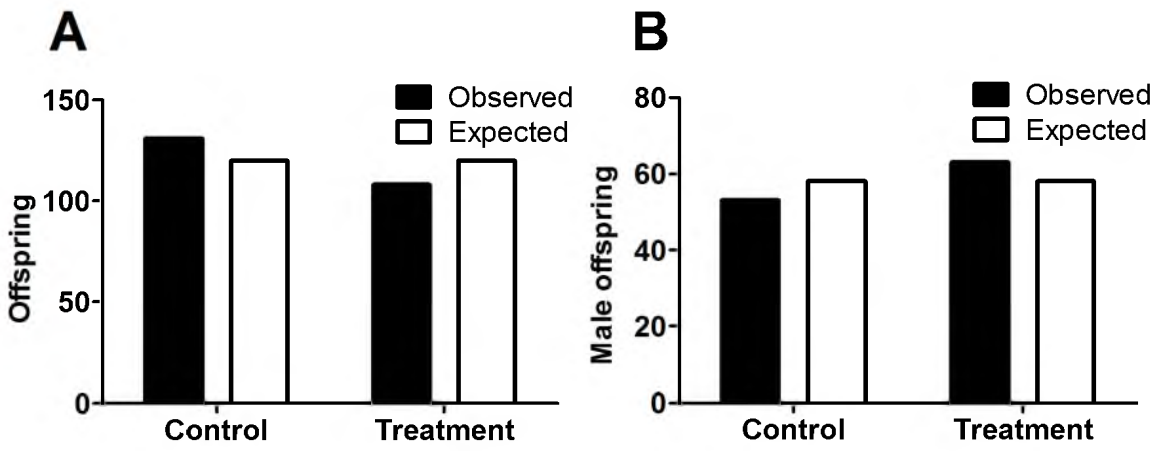

Figure 4.3. Reproduction of treatment and control animals by sex. Dendrimer exposure did not significantly impact female reproduction **a,** (Chi-square with Yates' correction; $\chi^2 = 0.927$, $P = 0.3356$). **b,** Likewise, no significant difference was seen in regards to male reproduction (Chi-square with Yates' correction; $\chi^2 = 0.2764$, $P = 0.5991$).

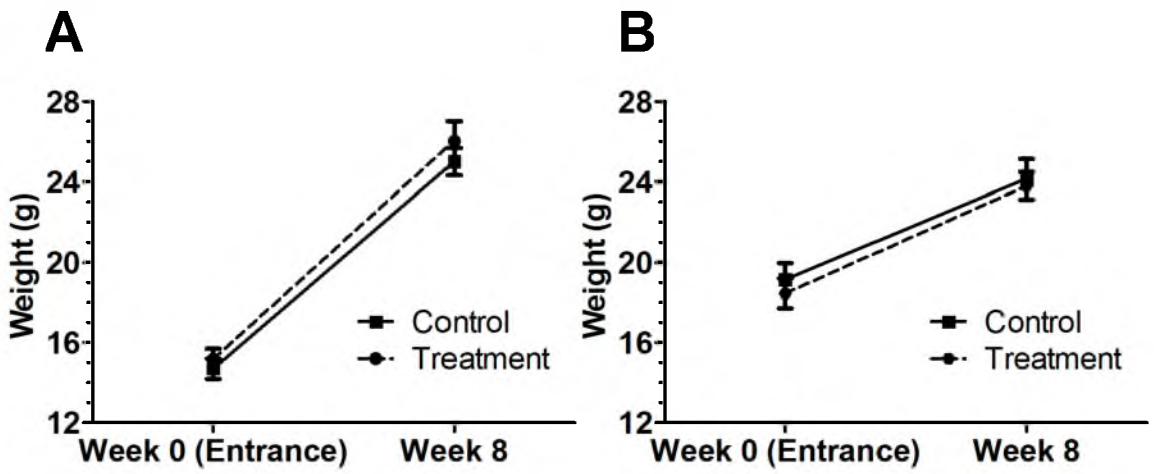

Figure 4.4. Mass of OPA founders at entrance and eight weeks later by sex. Dendrimer exposure did not significantly impact female (ANOVA; $F_{1,66} = 1.118$, $P = 0.2941$) **a,** or male (ANOVA; $F_{1,29} = 0.4195$, $P = 0.5223$) **b,** mass. For both sexes mass did significantly increase with time (ANOVA; $F_{1,66} = 224.6$, $P < 0.0001$), (ANOVA; $F_{1,29} = 38.80$, $P < 0.0001$).

Table 4.1. Summary of mixed model results for competitive ability.

Male Competitive ability	GLMM with binomial distribution and logit link (Intercept at week 0)			
Random effects	*Variance*	*Std. Deviation*		
Population (Intercept)	0.0000	0.0000		
Fixed effects	*Estimate*	*Std. Error*	*Z value*	*Pr(>|z|)*
Intercept	-1.7381	0.3833	-4.534	<0.0001***
Treatment	1.4574	0.4901	2.974	0.0029**
Time	0.0876	0.0313	2.794	0.0052**
Treatment*Time	-0.0568	0.0413	-1.375	0.1690

* Indicates a *P* value < 0.05, **< 0.01, ***< 0.001.

CHAPTER 5

MHC SIGNALING DURING SOCIAL COMMUNICATION

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CHAPTER 17

MHC SIGNALING DURING SOCIAL COMMUNICATION

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Abstract: The major histocompatibility complex (MHC) has been known to play a critical role in immune recognition since the 1950s. It was a surprise, then, in the 1970s when the first report appeared indicating MHC might also function in social signaling. Since this seminal discovery, MHC signaling has been found throughout vertebrates and its known functions have expanded beyond mate choice to include a suite of behaviors from kin-biased cooperation, parent-progeny recognition to pregnancy block. The widespread occurrence of MHC in social signaling has revealed conserved behavioral-genetic mechanisms that span vertebrates and includes humans. The identity of the signal's chemical constituents and the receptors responsible for the perception of the signal have remained elusive, but recent advances have enabled the identification of the key components of the behavioral circuit. In this chapter we organize recent findings from the literature and discuss them in relation to four nonmutually exclusive models wherein MHC functions as a signal of (i) individuality, (ii) relatedness, (iii) genetic compatibility and (iv) quality. We also synthesize current mechanistic studies, showing how knowledge about the molecular basis of MHC signaling can lead to elegant and informative experimental manipulations. Finally, we discuss current evidence relating to the primordial functions of the MHC, including the possibility that its role in social signaling may be ancestral to its central role in adaptive immunity.

Self and Nonself, edited by Carlos López-Larrea.

INTRODUCTION

MHC (also known as HLA in humans and H-2 in mice) signaling mediates both immune recognition during the adaptive immune response (discussed in the previous chapter) and social signaling that enhances both the recognition of optimal mates and kin-biased behaviors.[1] Social signaling meditated by the MHC was first discovered in regards to mate preferences in laboratory mice (*Mus musculus*),[2] a full three decades after the histocompatibility functions were described by George Snell.[3] Thirty years later, social signaling via MHC has been described throughout vertebrates including mammals, birds, reptiles, amphibians and teleost fish (see Table 1). MHC social signaling has been identified in over 20 species of vertebrates and is likely the basis for a vertebrate-wide chemosensory communication system. The original observation of MHC disassortative mating preferences seems to be common, but not omnipresent in vertebrates;[4] it by no means is the only behavior facilitated by MHC, nor is it the only type of observed MHC-based mate preference. MHC signaling also facilitates cooperative behavior with kin, parent-progeny recognition and pregnancy block. In the following sections we will present the current evidence for MHC as a signal of relatedness, individuality, genetic compatibility and quality. MHC-mediated behaviors are diverse and though general patterns exist within vertebrates, the exact function of MHC-based social signaling will be species specific and highly context dependant.

SIGNALING OF MHC GENOTYPE: MOLECULAR MECHANISMS

For three decades after the discovery of MHC-mediated social singling in laboratory mice,[2] the actual mechanism of how MHC genotype was perceived in conspecifics remained a mystery. Early on it was discovered that MHC genotype could be discriminated by chemical cues detected by the olfactory system. These studies showed that mice could discriminate MHC odortypes either through training[5] or in the absence of training.[6] However, the nature of the signaling odorants remained elusive. This mystery was at least partially solved by the discovery that peptides known to bind MHC molecules also bound receptors in the vomeronasal organ (VNO).[7] It was later shown that a similar process was working in the main olfactory epithelium (MOE).[8]

The critical role of MHC-presented peptides during adaptive immune recognition is well established.[9] MHC-bound peptides are presented at the cell surface for interrogation by T cells; when the peptides are of foreign origin (e.g., from a pathogen) an immune response is initiated. The majority of MHC alleles encode unique structural aspects of the peptide binding region of the molecule and these variants provide great specificity in the peptides they present. Because there is physical correspondence between MHC allelic variants and the anchor positions of the amino acid sequence of their bound peptides, it was hypothesized that MHC peptides could serve as ligands for odorant receptors that had similar binding specificity, thus allowing information about MHC genotype to be conveyed. Physiological recordings from vomeronasal sensory neurons (VSNs) stimulated with synthetic peptides proved this to be the case.[7]

Table 1. Summary of studies investigating MHC-genotype signaling in social communication

Species	MHC-Based Mate Preference	MHC-Mediated Cooperative Behavior	Phenotype Matching System	Sources
Mammals				
House Mice (*Mus musculus*)	MHC disassortative	Female Communal Nesting	Familial imprinting	Yamazaki et al 1976,[2] 1988,[20] 2007;[21] Manning et al 1992;[22]
Bank voles (*Clethrionomys glareolus*)	MHC disassortative	Unknown	Unknown	Radwan et al 2008[23]
Malagasy giant jumping rat (*Hypogeonys antimena*)	MHC assortative	Unknown	Unknown	Sommer 2005[24]
Humans (*Homo sapiens*)	MHC disassortative	Unknown	Unknown	Wedekind et al 1995;[25] Havlicek and Roberts 2008[26]
Mandrill (*Mandrillus sphinx*)	MHC disassortative	Unknown	Unknown	Setchell et al 2009[27]
Fat-tailed dwarf lemur (*Cheirogaleus medius*)	MHC supertype-disassortative and maximal diversity	Unknown	Unknown	Schwensow et al 2008[28]
Grey mouse lemur (*Microcebus murinus*)	MHC disassortative (cryptic)	Unknown	Unknown	Schwensow et al 2008[29]
Domestic sheep (*Ovis aries*)	No MHC preference			Paterson and Pemberton 1997[30]
Birds				
Savannah sparrows (*Passerculus sandwichensis*)	MHC disassortative	Unknown	Unknown	Freeman-Galant et al 2003[31]
House Sparrow (*Passer domesticus*)	MHC assortative and optimal diversity	Unknown	Unknown	Bonneaud et al 2006[32]
Seychelles warbler (*Acrocephalus sechellensis*)	MCH maximal diversity	Unknown		Richardson et al 2005[33]
Great reed warbler *(Acrosephalus arundinaceus*)	No MHC preference			H. Westerdahl 2004[34]
Red jungle Fowl (*Gallus gallus*)	MHC disassortative (cryptic)	Unknown	Unknown	Gillingham et al 2009[35]
Peafowel (*Pavo cristatus*)	MHC maximal diversity (cryptic)	Unknown		Hale et al 2009[36]

(continued on next page)

Table 1. Continued

Species	MHC-Based Mate Preference	MHC-Mediated Cooperative Behavior	Phenotype Matching System	Sources
Reptiles				
Sand lizards (*Lacerta agilis*)	MHC disassortative	Unknown	Unknown	Olsson et al 2003[37]
Tuatara (*Sphenodon punctatus*)	MHC disassortative	Kin avoidance during territory acquisition	Unknown	Miller et al 2009[38]
Amphibians				
African clawed Frog (*Xenopus laevis*)	Unknown	Tadpole schooling	Self reference	Villinger and Waldman 2008[39]
Tiger Salamanders (*Ambystoma tigrinum*)	MHC assortative	Unknown	Unknown	Bos et al 2009[40]
Fish				
Zebrafish (*Danio rerio*)	Unknown	Unknown but kin groups grow faster than non kin groups	Familial imprinting	Gerlach et al 2007[41] and 2008[42]
Three-spined stickleback (*Gasterosteus aculeatus*)	Optimal MHC diversity	Unknown	Self reference	Aeschlimann et al 2003;[43] Reusch et al 2001;[44] Milinski 2006[45]
Atlantic Salmon (*Salmo salar*)	MHC disassortative	Schooling with kin	Self reference	Rajakaruna et al 2006;[46] Consuegra and de Leaniz 2008[47]
Chinook salmon (*Oncorhynchus tshawytscha*)	MHC disassortative	Unknown	Unknown	Neff et al 2008[48]
Arctic Char (*Salvelinus alpinus*)	Unknown	Schooling with kin	Self reference	Olsen et al 2002[49]
Brown Trout (*Salmo trutta L*)	Optimal MHC diversity	Unknown	Self reference	Forsberg et al 2007[50]
Brook Trout (*Salvelinus fontinalis*)	Unknown	Schooling with kin	Self reference	Rajakaruna et al 2006[46]
Whitefish (*Coregonus sp.*)	No MHC preference (cryptic)			Wedekind et al 2004[51]

Sources are limited to first reports and reviews. Blank boxes indicate no finding would be expected given the observed result.

Detection of Peptides in the Olfactory System

The olfactory system of mammals is anatomically divided into two regions: the main olfactory epithelia (MOE) and the vomeronasal organ (VNO). Traditionally these two organs were viewed as functioning in largely non-overlapping modalities with the VNO being specialized for detection of nonvolatile small molecules and proteins that typically signaled the sexual and social status of conspecifics (pheromones), while the MOE was thought to specialize as a general detection system for volatile substances.

The initial experiments to determine if the olfactory system was capable of detecting peptides were conducted in the VNO of mice. Leinders-Zufall and coworkers (2004) tested the hypothesis that dissociated MHC class I peptides could be detected in the VNO Two peptides known to be presented either by the H-2D^b haplotype (AAPDNRETF) or H-2K^d haplotype (SYFPEITHI) were synthesized. These peptides were applied individually to ex-vivo preparations of mouse VNO. Both peptides activated a relatively specific subset of V2R-positive neurons in the basal zone of the VNO as revealed by extracellular field potential recordings and fluorescence imaging. The VSNs responded with high sensitivity at concentrations down to 10^{-12}M.

As predicted by the hypothesis that peptides can signal MHC genotype, the peptide binding by the VSNs responded in an MHC allele-specific manner. Not only was the VSN response specific to the amino acid sequence of each peptide, but the pattern of specificity mimicked the binding properties of MHC molecules. Amino acid substitutions (underlined) at non-anchor positions (e.g., SYIPSAEKI) usually continued to stimulate the same neurons. In contrast, substitution of peptide anchor residues (underlined) with alanine (e.g., AAPDARETA or SAFPEITHA) abolished stimulation of these neurons. These VSN binding properties provide a neurophysiological basis for identifying the MHC genotype of individuals, because peptides are reverse-image "molds" of the antigen-binding site of MHC molecules. Thus, sensory receptors that detect peptides in an MHC-like fashion could in principle function as an MHC genotyping system.[10] These results point to the structural importance of peptide anchor residues in binding VSN receptors and, given the similar binding properties of MHC molecules, reveal the convergent ligand-binding properties of these unrelated molecules.

The same lab group applied the same hypotheses to the MOE sensory neurons, traditionally viewed as generalist receptors of volatile chemosignals.[8] Contrary to conventional wisdom, they discovered that nonvolatile, fluorescent tagged MHC peptides gain access to the MOE without direct nasal contact to the peptide containing fluid. Most importantly, these peptides activated neurons at subnanomolar concentrations in an allele specific fashion, similar to the patterns found in the VNO. There are, however, some important physiological differences in peptide detection between the two olfactory organs.[11] First, a different transduction mechanism is used in the MOE during recognition of peptides.[12] Second, when anchor residues are substituted with alanine (eg. AAPDARETA and SAFPEITHA), olfactory sensory neurons (OSNs) cease firing at normal stimulation concentrations, but firing resumes at higher concentrations. Third, MOE-dependent peptide recognition does not induce pregnancy block,[13] despite normal MHC odor (mating) preferences. These experiments show that discrimination of MHC genotype by the two olfactory systems is achieved with separate neurological, physiological and behavioral response pathways.

If peptides are the odorants that allow MHC genotype to be discriminated, then experimental manipulation of peptides should alter behavioral responses in a fashion

consistent with MHC-mediated behaviors. The findings that the MOE and the VNO can detect peptides in an MHC-like fashion stimulated research confirming that both mouse and stickleback fish (*Gasterosteus aculeatus*) behavior is manipulated by the addition of peptides. The experimental addition of peptides to an MHC similar odor source causes animals to respond as if it were an MHC dissimilar odor source for both mating- and odor-preferences[8,14] and pregnancy block.[7]

Signaling of MHC Genotype without Peptides

Due to the general nonvolatility of peptides,[15,16] the question has remained whether peptides can explain all of the observed MHC-mediated behavioral patterns. This question was recently addressed by experimentally removing all of the peptide components from the urine of two MHC-congenic strains of mice. Mice that had been trained to discriminate between the urine odors of these two strains could continue to discriminate using the peptide-free urine.[17] These results suggest that nonpeptide volatile odorants also provide signals conveying MHC genotype information. However, odor-training experiments can introduce confounding behavioral artifacts[18] and this result should be confirmed in a paradigm that does not use training. If these results are confirmed, making yet a third independent mechanism for identifying MHC genotype, it underscores the functional importance of this olfactory ability and the importance of the associated behavioral responses.

Though it has been shown that peptides signal MHC genotype in mammals (mice) and fish (sticklebacks), the utilization of peptides in other vertebrates is undocumented. It has been questioned weather olfaction can explain MHC-mediated behavior in birds whose olfactory prowess has long been questioned.[19] No other mechanisms have been as thoroughly tested as peptide and volatile olfaction signaling of MHC genotype and more work is needed to test whether these mechanisms drive MHC mediated behavior in other taxa.

MHC AS A SIGNAL IN INDIVIDUAL RECOGNITION

Individual recognition is an important component of social behavior. Traits that specifically signal individual identity are predicted to be genetically determined, highly variable, cheap to produce (i.e., not condition-dependent) and signal variants are expected to have equal fitness at equilibrium (reviewed in Tibbetts and Dale 2007 ref. 52). MHC is an ideal candidate gene for understanding the mechanistic bases of individual recognition because it is a genetically determined trait associated with social behavior and is extremely variable (there are 10^9 MHC phenotypes in mice[53]). MHC was hypothesized to contribute to individual recognition as early as 1975.[54] Since then, the concept of individual recognition has been invoked in many studies addressing MHC-associated cues in social signaling (e.g., ref. 17). However, many authors tacitly use different definitions of this term and do not distinguish between individual recognition in the strict sense[52] and other forms of social recognition, which can include discrimination of familiar vs unfamiliar conspecifics, kin vs nonkin, same-genotype vs different-genotype and genetically compatible vs incompatible mates. We define individual recognition as being characterized by individual specificity in three elements of social communication: signaling; signal perception and template matching by the signal receiver; and a functional response by the receiver.[55] This definition includes any case where receivers have a template of a specific individual

based on a learned signal and differs from kin-recognition where the template is based on phenotype matching (see below). Here, we review studies that have sought to characterize individual-specific MHC odortypes, which have mainly focused on MHC-correlated volatile profiles and their relation to pregnancy block and scent marking.

MHC congenic strains of mice, which share the same background genome, but have unique MHC haplotypes, are a model system with which to understand behavioral responses to individuals of same- or different-haplotype at a single locus. One extrapolation from studies demonstrating MHC haplotype-dependent behavior[17] in congenic strains is the possibility that, in outbred populations where MHC allelic polymorphism is likely to be very high, MHC phenotypes would be key mediators of individual recognition. For example, it has long been understood that MHC congenic strains have unique volatile organic compound signatures that are used in chemical communication.[15] More recently, several groups have identified suites of volatile organic compounds that are regulated by MHC odortypes.[15,16,56] As predicted by a model of individual recognition, some of these suites are unaffected by environmental variation;[57] furthermore, volatile profiles from MHC congenic mice activate overlapping but distinct subsets of neurons in the mouse main olfactory bulb.[58] The authors of such studies in congenic strains often conclude that the physiological machinery is in place for volatile profiles to mediate individual-specific behaviors (e.g., ref. 57). However, counter-part experiments using outbred wild mice in a more ecologically realistic setting are lacking. Given that some genotypes will inevitably be shared between individuals, more naturalistic work is needed to understand how these volatile signatures function as signals of individuality (as defined above) or as signals of relatedness or genotype.

Pregnancy Block

Pregnancy block, also known as the Bruce effect, occurs when recently mated female laboratory mice are exposed to the odors of an unfamiliar male.[59] Upon exposure to an unfamiliar male odor, prolactin release from the anterior pituitary in the mated female is suppressed, resulting in pregnancy failure, reabsorption of the fetus and the onset of estrus.[60] The signal responsible for pregnancy block is considered to be individual specific because the unfamiliar male and the mate both express odors capable of inducing pregnancy block. Thus, females have to learn the identity of their mate (i.e., form a memory) in order to suppress pregnancy block upon perception of the mate's odors.

Pregnancy block can be induced by the presence of an unfamiliar male or simply his soiled bedding or urine and direct physical contact with the odorant seems necessary.[60] However, in at least one case volatiles alone (i.e., no direct contact) can induce pregnancy block.[61] The memory developed during pregnancy block is dependent on activation of sensory neurons in the VNO; however, the specific chemical constituents that bind receptors in these neurons have proven difficult to find. Three different classes of molecules associated with individual odors have recently been investigated: MHC and MHC peptides, major urinary proteins (MUPs) and volatiles. Peele and colleagues recently investigated the relative roles of MUPs and volatiles.[62] They found that low molecular weight fractionations (which excludes MUPs) from urine were more effective in blocking pregnancy than those of high molecular weight, suggesting a role of volatile compounds in the odor. However, the low molecular weight fraction from the unfamiliar male resulted in only 50% pregnancy block, as opposed to 90% pregnancy block via unfamiliar male whole urine. Moreover, a recent study called these findings into doubt by showing that,

contrary to Hilda Bruce's original finding, urine from castrated or juvenile males was sufficient to induce pregnancy block. These results suggest that, although volatiles can contribute to the occurrence of pregnancy block, they are not necessary to induce it.[63]

MHC-associated odors have also been shown to be sufficient to induce pregnancy block in several studies, implicating it's involvement during individual recognition. These odors were originally observed to block pregnancy when unfamiliar males differing only at the MHC could induce pregnancy block.[61] Since then, searches for an MHC-odortype mechanism have targeted MHC molecules themselves, MHC peptides and possible associated volatiles. MHC peptides were the first specific odorant found to induce pregnancy block[7] (see above).

The finding that sensory neurons in the VNO respond selectively to MHC peptides was biologically validated by demonstrating the role of peptides in producing pregnancy block.[7] As predicted, it was found that pregnancy block upon exposure to MHC peptides from an unfamiliar, MHC-dissimilar male was equally effective as exposure to whole urine from an unfamiliar, MHC-dissimilar male. In this case, the peptides had to be delivered on a urinary background (regardless of whether the urine was from a familiar or unfamiliar male). A more recent study, however, found that peptides alone (administered more frequently than in ref. 7) were sufficient to induce pregnancy block.[63] These studies show that the suite of peptides presented by an individual's MHC molecules can, when excreted in urine, be used as odorants in chemical signaling. Because of the large diversity of MHC haplotypes in a population, there is potential for individual specific odortypes simply in excreted MHC peptides. Such odortypes are detectable by VSNs that have binding specificity for these peptides similar to that of MHC molecules.[7] Where these peptide signals originate, however, remains to be found. Surprisingly, there is disagreement about whether peptides can be found in mouse urine.[7,60,64] Peptides have not been reported in other mediums of chemical communication such as saliva, tears, or skin excretions, but we are not aware of any directed searches for peptides in these secretions.

Although MHC peptides are clearly sufficient to induce pregnancy block in inbred mice, it should be noted that the experiments described above do not demonstrate individual recognition in a strict sense. Because peptides from an unfamiliar male with the same MHC genotype as the female's mate would not be expected to induce pregnancy block, MHC peptides in the context of pregnancy block might be more likely to signal the presence of an unfamiliar male. If individuality is perceived during pregnancy block, it would likely be conveyed via coupling with sensory neurons activated by the urinary background and neurons in the VNO have been found to be capable of discriminating individual mice of the same laboratory strain.[64] Finally, while pregnancy block provides an attractive system in which to test hypotheses concerning social signaling and behavior, the system is ultimately hindered by the fact that the adaptive significance of pregnancy block, which is only observed in certain laboratory strains of mice, has not been determined for natural populations. It has been suggested that the Bruce effect functions to prevent infanticide from males who have recently displaced the dominant, territorial male.[4,65]

Scent-Marking

In addition to the MHC, growing evidence indicates that major urinary proteins (MUPs) are another chemical signal critical to social communication and individual recognition in mice. MUPs are protein pheromones encoded by a polymorphic, multi-gene family. In a series of experiments, the laboratory of Jane Hurst has tested the relative

roles of MUPs and MHC in individual recognition in mice using a scent-marking behavioral paradigm. First, it was shown that wild-derived males presented with a scent mark from another male expressing a different MUP-type will investigate and counter-mark the marks significantly more than the control.[66] Second, it was shown that scent-marks associated with MHC haplotype (in MHC-congenic strains) were not necessary or sufficient to influence investigation time of male mice of congenic MHC strains. Rather, investigation time was increased only when the stimulus odor differed from the genomic background of the test animal.[67] A third experiment tested whether wild female mice could discriminate between scent marks from congenic males whose MHC and MUP genotype were controlled. Results showed that females could discriminate between individual males only when the males differed with respect to MUP haplotype; females could not discriminate between individual males that had the same MUP haplotype and could not discriminate between males that had different MHC haplotypes.[68] These three experiments indicate that, in the context of scent-marking and countermarking, MUP genotype and not MHC genotype, is the greatest determinant of individuality in urinary odors. However, it should be noted that in light of previous research, it is anomalous that the mice in these experiments did not discriminate between urinary odors that differed with respect to MHC genotype.[67,68] Previous studies have documented the ability of either MHC-congenics (e.g., ref. 5) or wild-derived mice[69] to distinguish urinary odors that only differed genetically at the MHC.

Because MUPs are likely to be polygenic, polymorphic signals only in a few rodent species it is unlikely that the functions discovered in *Mus* will have generality across vertebrates. The results from the Hurst group studies suggest that there are key differences in signals that are conserved across taxa (e.g., MHC) and signals that are species-specific (e.g., MUPs) for the identification of individual conspecifics.[68] They also reveal the curious finding that signals of individuality are limited to specific behavioral interactions.

Taken together, the individual recognition studies reviewed above show that MHC may play an important role in individual recognition in certain instances (for example in pregnancy block), but also indicate that they may not be used for individual recognition in the strict sense. Many of the studies focusing on individual recognition and the MHC have utilized congenic strains of mice, which provide a unique opportunity to study the role of a single locus or haplotype in chemical communication. However, the use of inbred stains of animals may limit our broader understanding of behavior and ecology, as 60 years of domestication has modified their behavior.[70] So, more studies will be needed to determine the role of MHC in individual recognition in outbred populations; we know of no such examples except for the aforementioned examples from the Hurst lab.

MHC AS A SIGNAL IN KIN RECOGNITION

Kin recognition using polymorphic genetic systems allows individuals to engage in behaviors specific to kin or nonkin. An individual's fitness is a product of both its own reproductive success (i.e., direct fitness) and the reproduction of close relatives (i.e., indirect fitness); thus, proper identification of kin facilitates cooperation (or at least decreased antagonism) with relatives and promotes behaviors that increase fitness.[71] Additionally, recognition of kin allows for the prevention of inbreeding, and therefore reduces the homozygous expression of deleterious recessive alleles. In order for a genetic system to be used accurately to recognize kin, it must contain enough allelic polymorphism to allow

discrimination between related and unrelated individuals. Kin recognition systems that can discriminate among a range of different-degree relatives have been reported.[72] MHC is the most polymorphic genetic system in vertebrates[4] and has long been considered to play a role in kin recognition by mediating cooperation,[1] parent-offspring identification[73] and mating preferences that prevent inbreeding.[74]

Two major phenotype matching mechanisms exist for MHC-based kin recognition within vertebrates (Fig. 1). The first is a self reference system in which individuals use their own MHC odortype as a template to recognize other individuals as kin.[39,43,46,49,50] The second is familial imprinting where individuals imprint upon the MHC odortypes of kin early in development and afterwards apply the learned MHC signals to unfamiliar individuals.[20,42,75] The degree to which familial imprinting and self reference systems identify kin differ remarkably (Fig. 1). Only familial imprinting systems can identify kin that do not share odortypes with a focal individual. However, the ability to recognize kin that do not share odortypes also allows for the false recognition of unrelated individuals as relatives; this could occur in mixed litters where odortypes produced by half siblings are based on haplotypes from an unrelated individual. Both phenotype matching systems can be used to identify kin through odortypes based on either specific MHC haplotypes (both haplotypes providing a specific odor) or by odortypes based on a blended odor of both haplotypes. For example self reference systems recognize either 25 or 75% of full siblings depending on whether specific haplotypes are recognized or only the genotypic odor of the blended haplotypes[76] (Fig. 1). Currently few studies have been conducted to determine the specifics of phenotype matching systems used in nature and more research is needed to determine the relative prevalence of familial imprinting vs self reference systems and the nature of the odortypes (specific haplotypes or blended genotypes) used. Interestingly, the two systems most described in nature are familial imprinting on haplotypes and self reference based on blended genotype odors which are the best and worst of the theorized kin recognition systems respectively (Fig. 1). Regardless of the phenotype matching system used, kin recognition is likely one of the major functions of MHC-mediated signaling and the very existence of familial imprinting is evidence supporting this hypothesis because kin recognition is the only function that is enhanced by familial imprinting; self reference will be superior for functions involving genetic compatibility, individuality, or quality.[65]

Phenotype matching systems can identify more kin if multiple polymorphic unlinked loci are used, presuming a match at any locus is a signal of relatedness.[77] Though the impact of multiple unlinked loci has minimal impact on familial imprinting systems it has profound consequences on self reference systems, where multiple loci dramatically improve kin recognition (Fig. 1). Within both teleost fishes and amphibians, taxa where self reference systems are common, the MHC is not inherited as a single unit but rather as two or four separate unlinked loci.[78,79] Whether this is coincidence or represents evidence that the inefficiency of self reference systems favors translocations that breakup the MHC linkage group will await more phylogenetic data. Within both teleost fishes and amphibians it has been shown that MHC Class II genes are sufficient, but not necessary, for kin recognition. It has been proposed that other unlinked MHC genes provide additional information used in kin recognition.[46,49] Likewise, in house mice it has been observed that when MHC signals of relatedness are controlled for, signals from a different polymorphic locus (MUPs, see below) can also be used as signals of relatedness. In nature, it is highly likely that both MUPs and MHC are utilized for kin recognition in tandem.[80]

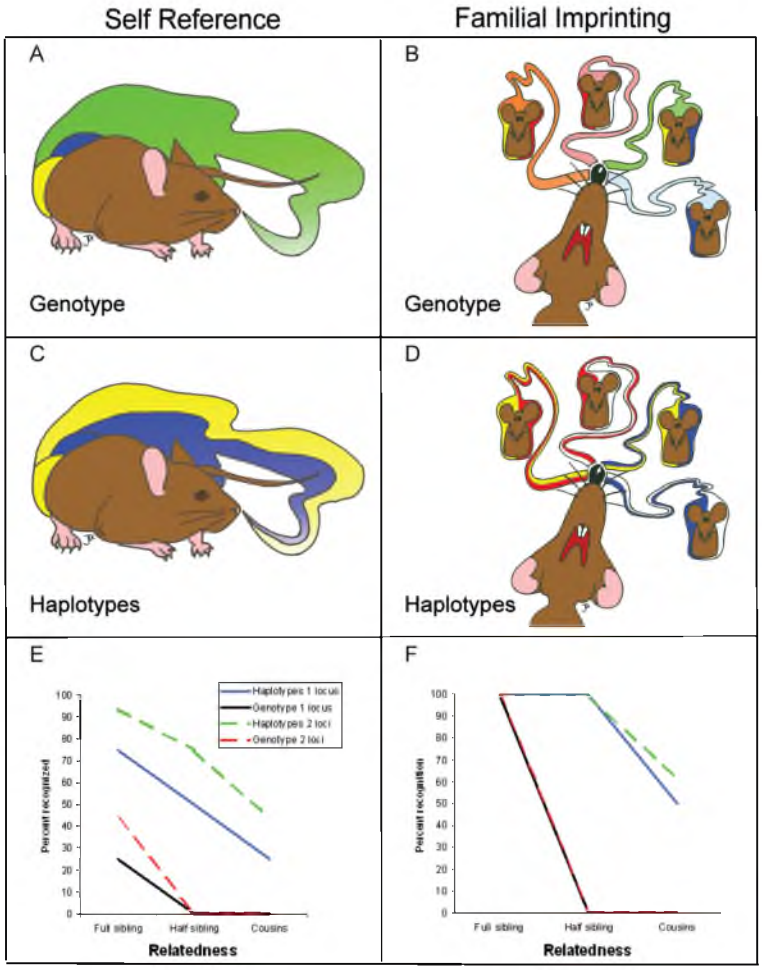

Figure 1. Possible phenotype matching systems using MHC-based odors and their effectiveness for the recognition of kin. Two kin recognition mechanisms that exist in nature are self reference (A, C, E) and familial imprinting (B,D,F). Phenotype matching can be based on genotypes (i.e. blended haplotype odor), or on haplotypes (i.e. allele-specific odors). Self reference is based on odors associated with an individual's own genotype (A) or both haplotypes (C). Familial imprinting is based on odors associated with the genotypes (B) or haplotypes (D) present in the natal nest (e.g. parents or siblings). The prevalence of these systems in nature is largely untested; current evidence suggests that the primary phenotype matching system in mice is haplotype-based familial imprinting (D). The effectiveness of each phenotype matching system for recognizing three classes of kin are plotted for one or two unlinked polymorphic loci (E & F). MHC haplotypes are inherited as a linked locus or as multiple unlinked loci depending on taxa. Each point represents the percentage of full siblings, half siblings, and cousins an individual would be able to recognize (points are connected by lines to help visualize patterns). Haplotype-based mechanisms are almost always superior to genotype mechanisms for kin recognition. Adding loci to self reference systems improves kin recognition more than in familial imprinting systems. Familial imprinting (F) generally allows an individual to recognize more kin than self reference (E). Models assume that all individuals are heterozygous, that no alleles are shared between unrelated individuals and that all combinations of parental genotypes are found within litters. (Illustrations by J.L.K; graphic design by Linda Morrison).

Cooperative Behavior

Proper identification of kin can result in cooperative behaviors between relatives; MHC mediated signaling has been shown to both promote cooperation and deter antagonism between individuals (Table 1). Schooling is an important cooperative behavior in fish and tadpoles that results in enhanced foraging and predator avoidance. Several salmonid species along with the African clawed frog (*Xenopus laevis*) have been shown to preferentially form schools with relatives that share MHC haplotypes[39,46,49] and it has been previously shown that kin-based schools have higher survival rates and larger territories.[81] A second MHC-mediated cooperative behavior has been documented in house mice; female mice communally nest and nurse offspring and it has been demonstrated that females preferentially nest with familiar sisters. When no familiar sisters are available, they preferentially nest with MHC-similar females.[1] Finally, competition over territories is fierce in many species of vertebrates and can result in serious injury; evidence suggesting that MHC signaling prevents territorial competition between kin has recently been demonstrated in tuataras (*Sphenodon punctatus*).[38] Scores of other kin-based cooperative behaviors have been documented within vertebrates and it is quite probable that we have only just begun to document those that are mediated by MHC signaling; however, it is not our intent to imply that all cooperative behaviors will be MHC-mediated. In fact, the precision of kin recognition systems will be enhanced as more polymorphic systems are used in signaling.

Parent-Progeny Recognition

Parent-progeny recognition prevents the expense associated with parental investment into unrelated individuals. This is especially true under conditions of communal living or in systems that involve extra-pair matings. Under these circumstances an identification system that could ensure parental care was only provided to genetic offspring would be highly adaptive and many such systems have been documented.[82] Female house mice nest communally and are therefore at risk of providing parental care to unrelated pups. Yamazaki and others[73] showed that female house mice can identify pups with which they share an MHC haplotype from congenic pups (genetically identical individuals with the exception of MHC type). Pups at the age of 15-21 days were also capable of recognizing and preferring their parents bedding to that of a MHC dissimilar congenic individual. This preference was reversible by cross-fostering, again showing the role of familial imprinting within MHC signaling in house mice. Currently this study offers the only evidence that MHC-mediated signaling is involved in parent-progeny recognition and though it was conducted with inbred strains of mouse, it reveals the potential of MHC signaling in nature.

Inbreeding Avoidance

Degradation of fitness due to inbreeding is a result of increased homozygosity of deleterious recessive alleles that are identical by descent. These alleles combine more frequently when related individuals reproduce compared to outbred matings. Early assessment of the fitness costs of full-sibling level inbreeding within vertebrates (mice) have been conducted and early studies showed a 10% decline in litter size.[83,84] However, these experiments only measured litter size reductions and they failed to assess the fitness

consequences of the inbred progeny in their natural context. In an experiment where the fitness impacts of a single generation of full-sibling inbreeding were assessed under seminatural conditions, it was found that outbred male mice had five-fold higher fitness than inbred males, with the consequences effectively approaching lethality for inbred sons. Daughters suffered an additional 20% reduction in fitness compared to previous assessments.[85] Likewise cousin-level inbreeding was shown to reduce male fitness by 34% and when the infectious agent *Salmonella* was present in the populations, the fitness decline in males was 57%.[86] Since the true negative consequences of inbreeding were only revealed under direct competition within a seminatural environment, we now refer to this experimental system as a phenotron because it allows the observers "to see" the true fitness consequences (phenotype) of a treatment. Disassortative MHC-based mating preferences function as a mechanism of inbreeding avoidance due to their highly polymorphic nature. Only closely related individuals are likely to share MHC haplotypes; thus a mating preference for MHC-dissimilar individuals will decrease the likelihood of inbreeding. The extent to which inbreeding can be avoided is dictated by whether a self reference or familial imprinting mechanism is utilized by a particular species.[76]

An indirect piece of evidence supporting MHC haplotype based familial imprinting and inbreeding avoidance within house mice has come from a study by Sherborne and colleagues.[80] This experiment investigated the relative importance of MHC and MUPs in mediating inbreeding avoidance behavior and its conclusion was that MHC is not involved in inbreeding avoidance behavior. House mice were released into seminatural enclosures with only full-sibling and half-sibling counterparts; inbreeding avoidance was assessed by the proportion of full-sibling vs half-sibling matings and genetic analysis was used to determine if there was either an MHC or MUP-based signal mediating inbreeding avoidance. The data showed that although no full-sibling inbreeding avoidance occurred, mice sharing exact MUP genotypes avoided mating with each other. This led the authors to conclude that MUPs are exclusively responsible for inbreeding avoidance in house mice and that MHC plays no role. However, this conclusion is unwarranted due to a flaw in the experimental design. Specifically, prior to testing in seminatural enclosures, test animals had been caged (since birth) with other individuals that possessed MHC haplotypes that were present in the enclosures. This design unintentionally allowed MHC familial imprinting to occur on all of the tested haplotypes; thus, animals upon entering the enclosures found themselves surrounded by individuals that would all be recognized as relatives by MHC-based systems. This situation forced the mice to make mate choices based on other non MHC cues and they utilized MUPs, preferring to mate with individuals that did not share exact genotypes. These results suggest MUPs are utilized in mate choice, but contrary to the conclusions of the paper, the design does not allow for the exclusion of a role for MHC. Furthermore, MUP-based mating preferences are based on self reference and not familial imprinting,[80] thus they do not offer the same protection against inbreeding that familial imprinted MHC preferences do.

MHC AS A SIGNAL OF GENETIC COMPATIBILITY IN MATE CHOICE

Genetic compatibility, broadly defined, refers to the degree to which an organism's genes, (both within and between haploid genomes), interact to increase or decrease fitness. Consequences of genetic incompatibility include inviable offspring (e.g. between species mating), severely reduced fitness (e.g. inbreeding), and incremental degradation of fitness

associated with the combination of incompatible alleles (e.g. MHC homozygosity). The fitness consequences of genetic compatibility might be so severe that finding a mate with the "right genes" to compliments one's own genome provides more indirect benefits than finding the "best genes" within high quality individuals.[87] In order to make MHC-based mate choice (or gamete fusion[88,89]) decisions in regards to genetic compatibility, individuals must possess the means to assess their own MHC types (see section on phenotype matching systems above). MHC-mediated odors readily signal information about the genetic compatibility between mates, and MHC-disassortative mating preferences (Table 1) lead to the production of offspring with compatible genotypes both at the MHC and throughout the genome.[76] The mechanisms of MHC-mediated genetic compatibility described below are MHC heterozygote advantage, offspring harboring different MHC genotypes than their parents (moving target) and the avoidance of inbreeding.

Heterozygote Advantage/Superiority

MHC-disassortative mate preferences by their very nature produce MHC heterozygous offspring, which are hypothesized to have superior immunocompetence.[90,91] Multiple lines of evidence now support the fitness-enhancing role of MHC-heterozygosity.[92-98] It was initially argued that MHC heterozygotes would have an advantage (overdominance) because they could present a wider variety of peptide antigens to the immune system making them more likely than MHC-homozygotes to recognize and mount an immune response against disease-causing agents. However, this mechanistic hypothesis has largely been rejected since experimental infections with single pathogens reveal that heterozygotes do not generally have an advantage over both homozygotes.[99] An alternative mechanism postulated that heterozygote advantage emerges over multiple infections because resistance is generally dominant and heterozygotes will benefit from the resistance profile of each allele, which masks some of the susceptibilities of each allele. This hypothesis was experimentally confirmed by laboratory-based experiments using coinfections with parasites having opposite MHC resistance/susceptibility profiles, which demonstrated that heterozygotes are more fit than either homozygote.[99] Recent studies on wild salmon[100] and vole[101] populations demonstrate that MHC heterozygotes have increased fitness under natural conditions of multi-parasite infection as well. The fitness enhancing nature of MHC heterozygote advantage in laboratory and natural settings is an example of the adaptive significance of MHC mediated signaling.

Moving Target

In addition to heterozygote advantage, selection could also favor MHC-disassortative mate preferences if the offspring genotype provided a moving target against pathogen adaptation, causing pathogens adapted to either parent to be at a disadvantage in progeny that are MHC-dissimilar to both parents.[102] This hypothesis predicts that pathogens evolve to partially escape MHC-mediated immune recognition and that MHC-dissimilar offspring are more fit than their parents when challenged with parent-adapted pathogens. Like heterozygote advantage, mate choice decisions driven by moving target processes function to maximize genetic compatibility and are thus most effectively achieved using an MHC, self reference phenotype matching system.

Numerous examples highlight the capacity of pathogens to rapidly adapt to escape MHC-mediated immune recognition.[103-110] There has been one experimental study designed

to test the other prediction of the moving target hypothesis—that MHC-dissimilar offspring will be more fit than their parents when challenged with parent-adapted pathogens. MHC did influence the trajectory of adaptation by a fungal pathogen (*Cryptococcus neoformans*), but the large virulence increase in postpassage pathogen lines showed no specificity for the host MHC genotype of passage.[111] The most likely explanation is that this pathogen is a generalist that infects most birds and mammals. The passages in mice therefore selected for adaptations to "mouseness", which likely swamped any adaptations to MHC. Future passage studies should use pathogens specialized on the host of passage.

There is anecdotal evidence from human studies demonstrating the importance of offspring genetic diversity in reducing the probability of mother-to-child-transmission of chronic infectious disease agents (e.g., HIV-1[112,113]), and suggest that there would be a significant selective advantage to mate choices that promoted genetic diversity in offspring. There is also evidence linking increased HLA dissimilarity between mother and offspring with significantly reduced chances of vertical transmission of HIV-1[114,115]). The extent of pathogen adaptation during chronic infection of the parent and its impact on mother-to-child transmission dynamics was not addressed in the above studies. Despite this, they do support the possible role of MHC- disassortative mate preferences in producing offspring of higher quality that are more resistant to infection by chronic parasites of their parents.

Optimal MHC Heterozygosity

MHC-disassortative mate choice may carry a cost if maximal MHC diversity in offspring is not optimal. For instance, during the process of negative selection in the thymus, T cells with high affinity for MHC-peptide complexes are instructed to terminate themselves via apoptosis.[9] It follows then that MHC diversity may have an upper limit beyond which the fitness benefit of having multiple ways to present peptides from foreign invaders is offset by the cost of an increasingly limited T-cell repertoire.[116] If such a fitness cost exists, then it will have important implications on the evolution of MHC disassortative mating preferences. Indeed, it has been observed that individuals with intermediate versus maximal MHC diversity harbor lower parasite burdens in experimental infections.[117] Additionally, it was recently shown that intermediate and not maximal levels of MHC diversity lead to significantly higher lifetime reproductive success in stickleback offspring.[118] Thus, it seems that maximum MHC diversity can be a costly trait.

If intermediate rather than maximal MHC diversity is optimal then an MHC-typing system could allow individuals to "optimize" the MHC diversity within their offspring. Studies with sticklebacks have shown that females are in fact capable of such quantitative estimates of MHC diversity (also known as allele counting).[119] Additionally, by estimating the extent of intra-individual MHC class IIB allele diversity within a population, it was also demonstrated that individuals with intermediate rather than maximal MHC diversity were most frequent, indicating selection for intermediate levels of MHC diversity. Subsequent experimental findings in sticklebacks[43] and brown trout[50] suggest that much of the selection for individuals with intermediate MHC diversity derives from female preference for MHC-dissimilar mates. Together, these studies indicate that maximal MHC diversity is not always optimal and that female preference for MHC-dissimilar mates is a primary driving force behind selection for the production of individuals with intermediate rather than maximal MHC diversity.

Inbreeding Avoidance

Though inbreeding avoidance has already been covered within the kin recognition section it is important to stress that it also falls under the umbrella of MHC as a signal of genetic compatibility. In fact, inbreeding avoidance may be the single most adaptive result of MHC-disassortative mating preferences in many species of vertebrates, as both sibling and cousin level inbreeding have been found to have devastating effects on the fitness of offspring.[85,86] In addition, as covered in the evolution of MHC section below, growing evidence suggests that MHC mediated kin recognition to avoid inbreeding may have been the ancestral function of MHC molecules, which were later co-opted for use in the adaptive immune system.[120]

MHC AND SIGNALS OF QUALITY IN MATE CHOICE

In contrast to MHC-mediated signals that directly convey MHC genotype information (relatedness, compatibility or individuality), the disease resistance functions of MHC can also influence social signalling by modulating the expression of secondary sexual characters. Only high-quality, disease-resistant individuals should be able to invest in costly, sexually selected advertisements,[121] thus creating a correlation between MHC genotype and these condition-dependent traits (Table 2). By endowing an individual with genetic resistance to parasites, MHC genotype can indirectly influence signals of quality by allowing more physiological resources to be devoted to signaling rather than to the immune response.[122] von Schantz and colleagues[123] were the first to report an association between MHC and a sexually selected trait; they found that spur length in male pheasants (*Phasianus colchicus*) was correlated with fitness and dependent on MHC genotype. In a study on great snipes (*Gallinago media*), females preferred males carrying specific MHC allelic lineages. Males with these genotypes were also larger and females of this species are generally known to favor larger males.[124] A study in white-tailed deer (*Odocoileus virginianus*) found that MHC divergent heterozygous males had larger antlers and body size, which was correlated with lower abundance of abomasal nematodes.[122] Finally, a study

Table 2. MHC correlations with secondary sexual traits and mating preferences

Species	MHC Correlation with Mate Preference	MHC Correlation with Traits of Quality	Sources
Great snipe (*Gallinago media*)	MHC allele-specific preference	Body size	Ekblom et al 2004[124]
Peafowl (*Pavo cristatus*)	MHC heterozygosity	Train length	Hale et al 2009[36]
Pheasants (*Phasianus colchicus*)	MHC genotype	Spur length	von Schantz et al 1996[123]
White-tailed deer (*Odocoileus virginianus*)	MHC divergent heterozygotes	Antler and body size; reduced parasitism	Ditchkoff et al 2001[122]

on a canonical sexually selected trait, trains in male peacocks (*Pavo cristatus*), showed that the train length reflects genetic diversity at the MHC.[36] The above examples show that MHC-genotype can influence the expression of secondary sexual traits that are used as signals of quality. However, MHC-genotype itself is not necessarily used in the signal.

An alternative way that MHC-genotype can indirectly influence the expression of secondary sexual characteristics is if MHC social signals are themselves costly to produce. This hypothesis has recently been tested by the laboratory of Manfred Milinski, which identified the first example of condition-dependent MHC signaling.[125] They had previously shown that female three-spined sticklebacks prefer males with optimal, rather than maximal, MHC allelic differences (relative to her own genotype) and that this mate choice is mediated by excreted MHC peptides (discussed above).[14,44] Now, they show that females do not send this signal at all and that, remarkably, males only send this signal when they are in the reproductive state. These data suggest that MHC signaling is not simply a byproduct of MHC-peptide presentation, but that it is actively regulated in a fashion consistent with it being a costly signal. The authors suggest that shedding MHC-peptide complexes will create localized deficiencies of this critical immunological component and therefore represents a trade off between immune defense and social signaling.[125]

MHC-mediated signals of quality may allow an individual to gain either direct benefits for themselves or indirect genetic benefits for their offspring. Avoidance of parasitism is perhaps the most likely direct benefit of MHC-mediated mate choice. Social behaviors are an opportunity for parasites to transmit to new hosts; in turn, hosts will gradually develop behavioral mechanisms to avoid parasites.[126] Individuals of a particular MHC-genotype may be resistant to local parasites at any given time and choosing such an individual as a mate would provide a direct benefit of reduced risk of parasitism. Although there are several examples of mate choice for parasite-free individuals,[127-129] there are surprisingly few examples of studies that link MHC-dependent resistance to pathogens and subsequent mate choice.[117]

MHC EVOLUTION: WHAT ARE THE PRIMORDIAL FUNCTIONS?

Since the immune recognition function of MHC genes in adaptive immunity was discovered far earlier than MHC-mediated behaviors, and since it was so central to the complex system of vertebrate adaptive immunity, it was initially assumed that MHC-mediated behaviors were a derived function. However, Brown argued that since kin-selected behaviors (inbreeding avoidance and kin-biased cooperation) are present in the ancestral lineages leading to vertebrates and that adaptive immunity is a derived character in vertebrates, it is most parsimonious to hypothesize that MHC-mediated kin recognition functions were primordial.[74] This controversy continues to this day.

Boehm has recently written a tour-de-force, synthetic review that evaluates self and nonself recognition systems that exist across plants, fungi and animals, with a special emphasis on how quality recognition is maintained in the face of the rapid diversification of these highly polymorphic systems.[120] Quality control (the ability to accurately discriminate between self and nonself) is of particular importance in immune recognition systems that must achieve self tolerance to protect against auto-immune disease.[120,130]

Jawless fish are the one lineage of vertebrates that appear to have a non MHC based adaptive immune recognition system.[131,132] A high diversity of lymphocyte receptors in this group is created by combinatorial assembly of receptor modules, but the critical difference

from other vertebrates is that there is no junctional diversity created by mutagenic joining mechanisms.[133] Thus, the lymphocyte receptor repertoire for jawless fish is predictable and self tolerance could be achieved by Darwinian selection for self-compatible receptor modules.[120] In contrast, jawed vertebrates achieve higher lymphocyte receptor diversity by the mutagenic VDJ combinatorial joining process, which creates the problem of unpredictable receptor specificities that can lead to auto-immunity. These potentially harmful receptors are eliminated during the evaluation of lymphocytes receptors in the thymus of jawed vertebrates. Boehm argues that it seems unlikely that an MHC-peptide presentation system could emerge de-novo to create the modern jawed vertebrate immune recognition system, which allows self-tolerance in the face of somatic generation of unpredictable lymphocyte receptors. It would be far more likely that a pre-existing MHC-peptide kin recognition system could be co-opted for immune recognition.[120] Discovery of the MHC homologues and their function in jawless fish offers one of the most promising approaches for discriminating between these two hypotheses and identifying the primordial function of MHC genes. Tunicates (a close relative of vertebrates) have a highly polymorphic histocompatibility-type (fusion) locus that functions both in allo-recognition to control colony fusion and gamete fusion,[134] at least in some species.[135] It was thought that identifying the nature of this locus might clarify the early history of MHC genes. After a two-decade search the locus was identified to be a member of the immunoglobulin super family, but it appears to not have homology to MHC genes.[136-138] These findings further focus the search for primordial MHC functions towards jawless fish.

The facts that within vertebrates there are completely different mechanisms controlling adaptive immune recognition and that in tunicates histocompatibility functions are controlled by genes unrelated to vertebrate histocompatibility genes, highlight the evolutionary flexibility of how similar functions can be achieved through different genetic systems. It is currently difficult to discriminate between the different proposed primordial function of MHC genes. However, the initial assumption that immune recognition must be the primordial function of MHC genes, should no longer be the default assumption.

CONCLUSION

In this chapter we have demonstrated the significance of MHC signaling in regards to four aspects of social communication. First, studies in mice show that MHC peptides and to a lesser extent MHC-associated urinary odors, signal individuality in the context of pregnancy block. MHC does not signal individuality during mouse scent-marking, rather, a species specific signal (MUP) is used. Second, MHC as a signal of relatedness is found across vertebrates (Table 1) and plays a role in cooperation, parent-offspring identification and inbreeding avoidance via two different phenotype matching mechanisms: self reference or familial imprinting. Third, MHC signals are used to determine the genetic compatibility of a potential mate and can result in the production of heterozygous offspring. In some animals, mate choice for MHC compatibility is so finely tuned that they can optimize the degree of MHC heterozygosity in their offspring. Fourth, information regarding MHC genotype can be signaled indirectly through correlated characters (Table 2) and a recent study demonstrated that, at least within one species, MHC signaling itself may be condition-dependent and therefore a signal of individual quality. Taken together, these studies suggest that MHC-mediated signaling is conserved across vertebrates, but takes on unique functions depending on the life-history of a given species.

Appreciating the distinction between both modes of phenotype matching (self reference and familial imprinting) is paramount in understanding the role MHC-mediated signaling plays in social communication. Though substantial overlap in functionality exists between these phenotype matching systems, there are tradeoffs. Self reference systems facilitate mating preferences that generate offspring with an immunological advantage by allowing the assessment of genetic compatibility. Familial imprinting systems of phenotype matching facilitate the identification of siblings, half-siblings and cousins; in species where either cooperative behavior or avoiding inbreeding is important (e.g., communal nesting species or species that live in high-density populations), a familial imprinting system provides an advantage over a self reference system because self/nonself discrimination is not required to increase indirect fitness. That these two systems are differentially utilized by different groups of vertebrates highlights the highly context-dependent nature of social signaling. It is important to note, however, that phenotype matching mechanisms have been described in a relatively small number of species (Table 1) and more studies are needed.

The remarkable fact that a single genetic system controls major components of both immune recognition and social recognition begs the question of which recognition system constituted the primordial function of MHC genes. The convergent evolution of similar peptide binding properties of MHC, VSN and OSN receptor molecules provides the molecular basis by which MHC genotype influences both immune and social recognition; it also implies that these distinct receptor families have responded to selective pressures that required information regarding MHC genotype (bound peptides) be associated with discriminatory sensory systems. Finally, the ubiquitous presence of various modes of self versus nonself discrimination across all three domains of life, coupled with the derived nature of the adaptive immune system in vertebrates, further suggests that MHC-mediated social signaling evolved for the purpose of discrimination between conspecifics and could represent the ancestral state. Tracing the function of MHC molecules across vertebrate evolution holds the greatest promise of resolving the relative importance of immune versus social communication in MHC evolution.

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REFERENCES

1. Manning CJ, Wakeland EK, Potts WK. Communal nesting patterns in mice implicate MHC genes in kin recognition. Nature 1992; 360:581-583.
2. Yamazaki K, Boyse EA, Mike V et al. Control of mating preferences in mice by genes in the major histocompatibility complex. J Exp Med 1976; 144:1324-1335.
3. Snell GD. Methods for the study of histocompatibility genes. J Genet 1948; 49:87-108.
4. Piertney SB, Oliver MK. The evolutionary ecology of the major histocompatibility complex. Heredity 2006; 96:7-21.
5. Yamaguchi M, Yamazaki K, Beauchamp GK et al. Distinctive urinary odors governed by the major histocompatibility locus of the mouse. Proc Natl Acad Sci 1981; 78:5817-5820.
6. Singh PB, Brown RE, Roser B. MHC antigens in urine as olfactory recognition cues. Nature 1987; 327:161-164.

7. Leinders-Zufall T, Brennan P, Widmayer P et al. MHC class I peptides as chemosensory signals in the vomeronasal organ. Science 2004; 306:1033-1037.
8. Spehr M, Kelliher KR, Li XH et al. Essential role of the main olfactory system in social recognition of major histocompatibility complex peptide ligands. J Neurosci 2006; 26:1961-1970.
9. Murphy KP, Travers P, Janeway C et al. Immunobiology. New York: Garland Science, 2008.
10. Boehm T, Zufall F. MHC peptides and the sensory evaluation of genotype. Trends Neurosci 2006; 29:100-107.
11. Spehr M, Spehr J, Ukhanov K et al. Parallel processing of social signals by the mammalian main and accessory olfactory systems. Cell Mol Life Sci 2006; 63:1476-1484.
12. Kelliher KR, Spehr M, Li XH et al. Pheromonal recognition memory induced by TRPC2-independent vomeronasal sensing. Euro J Neuroscience 2006; 23:3385-3390.
13. Kelliher KR, Spehr M, Li XH et al. Relative roles of the main and accessory olfactory systems in behavioral responses to MHC class I peptides: Bruce effect. Chem Senses 2005; 30:A18.
14. Milinski M, Griffiths S, Wegner KM et al. Mate choice decisions of stickleback females predictably modified by MHC peptide ligands. Proc Natl Acad Sci USA 2005; 102:4414-4418.
15. Singer AG, Beauchamp GK, Yamazaki K. Volatile signals of the major histocompatibility complex in male mouse urine. Proc Natl Acad Sci USA 1997; 94:2210-2214.
16. Willse A, Belcher AM, Preti G et al. Identification of major histocompatibility complex-regulated body odorants by statistical analysis of a comparative gas chromatography/mass spectrometry experiment. Anal Chem 2005; 77:2348-2361.
17. Kwak J, Opiekun MC, Matsumura K et al. Major histocompatibility complex-regulated odortypes: peptide-free urinary volatile signals. Physiol Behav 2009; 96:184-188.
18. Carroll LS, Penn DJ, Potts WK. Discrimination of MHC-derived odors by untrained mice is consistent with divergence in peptide-binding region residues. Proc Natl Acad Sci USA 2002; 99:2187-2192.
19. Zelano B, Edwards SV. An Mhc component to kin recognition and mate choice in birds: Predictions, progress and prospects. Am Nat 2002; 160:S225-S237.
20. Yamazaki K, Beauchamp GK, Kupniewski J et al. Familial imprinting determines H-2 selective mating preferences. Science 1988; 240:1331-1332.
21. Yamazaki K, Beauchamp GK, Daisuke Y. Genetic Basis for MHC-Dependent Mate Choice. Adv Genet 2007; 59:129-145.
22. Manning CJ, Wakeland EK, Potts WK. Communal nesting patterns in mice implicate MHC genes in kin recognition. Nature 1992b; 360:581-583.
23. Jacek R, Aleksandra T, Agnieszka K. MHC and Preferences for Male Odour in the Bank Vole. Ethology 2008; 114:827-833.
24. Sommer S. Major histocompatibility complex and mate choice in a monogamous rodent. Behav Ecol Sociobiol 2005; 58:181-189.
25. Wedekind C, Seebeck T, Bettens F et al. MHC-dependent mate preferences in humans. Proc R Soc Lond B 1995; 260:245-249.
26. Havlicek J, Roberts SC. MHC-correlated mate choice in humans: A review. Psychoneuroendocrinology 2009; 34:497-512.
27. Setchell JM, Charpentier MJ, Abbott KM et al. Opposites attract: MHC-associated mate choice in a polygynous primate. J Evol Biol 2009; 23:136-148.
28. Schwensow N, Fietz J, Dausmann K et al. MHC-associated mating strategies and the importance of overall genetic diversity in an obligate pair-living primate. Evol Ecol 2008; 22:617-636.
29. Schwensow N, Eberle M, Sommer S. Compatibility counts: MHC-associated mate choice in a wild promiscuous primate. Proc R Soc Lond B Biol Sci 2008; 275:555-564.
30. Paterson S, Pemberton JM. No evidence for major histocompatibility complex-dependent mating patterns in a free-living ruminant population. Proc R Soc Lond B 1997; 264:1813-1819.
31. Freeman-Gallant CR, Meguerdichian M, Wheelwright NT et al. Social pairing and female mating fidelity predicted by restriction fragment length polymorphism similarity at the major histocompatibility complex in a songbird. Mol Ecol 2003; 12:3077-3083.
32. Bonneaud C, Chastel O, Federici P et al. Complex Mhc-based mate choice in a wild passerine. Proc Biol Sci 2006; 273:1111-1116.
33. Richardson DS, Komdeur J, Burke T et al. MHC-based patterns of social and extra-pair mate choice in the Seychelles warbler. Proc Biol Sci 2005; 272:759-767.
34. Westerdahl H. No evidence of an MHC-based female mating preference in great reed warblers. Molecular Ecology 2004; 13:2465-2470.
35. Gillingham MAF, Richardson DS, Lovlie H et al. Cryptic preference for MHC-dissimilar females in male red junglefowl, Gallus gallus. Proc R Soc Lond B Biol Sci 2009; 276:1083-1092.
36. Hale ML, Verduijn MH, Moller AP et al. Is the peacock's train an honest signal of genetic quality at the major histocompatibility complex? J Evol Biol 2009; 22:1284-1294.

37. Olsson M, Madsen T, Nordby J et al. Major histocompatibility complex and mate choice in sand lizards. Proc R Soc Lond B Biol Sci 2003; 270:S254-S256.
38. Miller HC, Moore JA, Nelson NJ et al. Influence of major histocompatibility complex genotype on mating success in a free-ranging reptile population. Proc Biol Sci 2009; 276:1695-1704.
39. Villinger J, Waldman B. Self-referent MHC type matching in frog tadpoles. Proc Biol Sci 2008; 275:1225-1230.
40. Bos DH, Williams RN, Gopurenko D et al. Condition-dependent mate choice and a reproductive disadvantage for MHC-divergent male tiger salamanders. Molecular Ecology 2009; 18:3307-3315.
41. Gerlach G, Hodgins-Davis A, MacDonald B et al. Benefits of kin association: related and familiar zebrafish larvae (Danio rerio) show improved growth. Behav Ecol Sociobiol 2007; 61:1765-1770.
42. Gerlach G, Hodgins-Davis A, Avolio C et al. Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. Proc Biol Sci 2008; 275:2165-2170.
43. Aeschlimann PB, Haeberli MA, Reusch TBH et al. Female sticklebacks Gasterosteus aculeatus use self reference to optimize MHC allele number during mate selection. 2003; 54:119-126.
44. Reusch T, Haeberli MA, Aeschlimann PB et al. Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. Nature 2001; 414:300-302.
45. Milinski M. The major histocompatibility complex, sexual selection and mate choice. Annu Rev Ecol Evol Syst 2006; 37:159-186.
46. Rajakaruna RS, Brown JA, Kaukinen KH et al. Major histocompatibility complex and kin discrimination in Atlantic salmon and brook trout. Mol Ecol 2006; 15:4569-4575.
47. Consuegra S, Garcia de Leaniz C. MHC-mediated mate choice increases parasite resistance in salmon. Proc Biol Sci 2008; 275:1397-1403.
48. Neff BD, Garner SR, Heath JW et al. The MHC and nonrandom mating in a captive population of Chinook salmon. Heredity 2008; 101:175-185.
49. Olsen KH, Grahn M, Lohm J. Influence of MHC on sibling discrimination in Arctic char, Salvelinus alpinus (L.). J Chem Ecol 2002; 28:783-795.
50. Forsberg LA, Dannewitz J, Petersson E et al. Influence of genetic dissimilarity in the reproductive success and mate choice of brown trout—females fishing for optimal MHC dissimilarity. J Evol Biol 2007; 20:1859-1869.
51. Wedekind C, Walker M, Portmann J et al. MHC-linked susceptibility to a bacterial infection, but no MHC-linked cryptic female choice in whitefish. J Evol Biol 2004; 17:11-18.
52. Tibbetts EA, Dale J. Individual recognition: it is good to be different. Trends Ecol Evol 2007; 22:529-537.
53. Singh PB. Chemosensation and genetic individuality. Reproduction 2001; 121:529-539.
54. Thomas L. Symbiosis as an immunologic problem. The immune system and infectious diseases. In: Neter E, Milgrom F, eds. Fourth International Convocation of Immunology. Basel: S. Karger; 1975:2-11.
55. Tibbetts EA, Sheehan MJ, Dale J. A testable definition of individual recognition. Trends Ecol Evol 2008; 23:356-356.
56. Novotny MV, Soini HA, Koyama S et al. Chemical identification of MHC-influenced volatile compounds in mouse urine. I: Quantitative proportions of major chemosignals. J Chem Ecol 2007; 33:417-434.
57. Kwak J, Willse A, Matsumura K et al. Genetically-based olfactory signatures persist despite dietary variation. PLoS One 2008; 3:e3591.
58. Schaefer ML, Young DA, Restrepo D. Olfactory fingerprints for major histocompatibility complex-determined body odors. J Neurosci 2001; 21:2481-2487.
59. Bruce HM. An exteroceptive block to pregnancy in the mouse. Nature 1959; 164:105.
60. Brennan PA. Outstanding issues surrounding vomeronasal mechanisms of pregnancy block and individual recognition in mice. Behav Brain Res 2009; 200:287-294.
61. Yamazaki K, Beauchamp GK, Wysocki CJ et al. Recognition of H-2 types in relation to the blocking of pregnancy in mice. Science 1983b; 221:186-188.
62. Peele P, Salazar I, Mimmack M et al. Low molecular weight constituents of male mouse urine mediate the pregnancy block effect and convey information about the identity of the mating male. Eur J Neurosci 2003; 18:622-628.
63. Thompson RN, McMillon R, Napier A et al. Pregnancy block by MHC class I peptides is mediated via the production of inositol 1,4,5-trisphosphate in the mouse vomeronasal organ. J Exp Biol 2007; 210:1406-1412.
64. He J, Ma L, Kim S et al. Encoding gender and individual information in the mouse vomeronasal organ. Science 2008; 320:535-538.
65. Slev PR, Nelson AC, Potts WK. Sensory neurons with MHC-like peptide binding properties: disease consequences. Curr Opin Immunol 2006; 18:608-616.
66. Hurst JL, Payne CE, Nevison CM et al. Individual recognition in mice mediated by major urinary proteins. Nature 2001; 414:631-634.
67. Hurst JL, Thom MD, Nevison CM et al. MHC odours are not required or sufficient for recognition of individual scent owners. Proc Biol Sci 2005; 272:715-724.

68. Cheetham SA, Thom MD, Jury F et al. The genetic basis of individual-recognition signals in the mouse. Curr Biol 2007; 17:1771-1777.
69. Penn D, Potts W. Untrained mice distinguish MHC-determined odors. Physiol Behav 1998; 64:235-243.
70. Manning CJ, Potts WK, Wakeland EK et al. What's wrong with MHC mate choice experiments? In: Doty RL, Müller-Schwarze D, eds. Chemical Signals in Vertebrates. Vol VI. New York: Plenum; 1992; 229-235.
71. Hamilton WD. The genetical evolution of social behaviour. I II J Theor Biol 1964; 7:1-52.
72. Mateo JM. Kin-recognition abilities and nepotism as a function of sociality. Proc Biol Sci 2002; 269:721-727.
73. Yamazaki K, Beauchamp GK, Curran M et al. Parent-progeny recognition as a function of MHC odortype identity. Proc Natl Acad Sci USA 2000; 97:10500-10502.
74. Brown JL. Some paradoxical goals of cells and organisms: the role of the MHC. In: Pfaff DW, ed. Ethical Questions in Brain and Behavior. New York: Springer Verlag; 1983; 111-124.
75. Penn D, Potts W. MHC-disassortative mating preferences reversed by cross-fostering. Proc R Soc London B 1998d; 265:1299-1306.
76. Potts WK, Wakeland EK. Evolution of MHC genetic diversity: a tale of incest, pestilence and sexual preference. Trends Genet 1993; 9:408-412.
77. Paterson S, Hurst JL. How effective is recognition of siblings on the basis of genotype? J Evol Biol 2009; 22:1875-1881.
78. Sato A, Figueroa F, Murray BW et al. Nonlinkage of major histocompatibility complex class I and class II loci in bony fishes. Immunogenetics 2000; 51:108-116.
79. Liu Y, Kasahara M, Rumfelt LL et al. Xenopus class II A genes: studies of genetics, polymorphism and expression. Dev Comp Immunol 2002; 26:735-750.
80. Sherborne AL, Thom MD, Paterson S et al. The genetic basis of inbreeding avoidance in house mice. Curr Biol 2007; 17:2061-2066.
81. Brown GE, Brown JA. Do kin always make better neighbours? The effects of territory quality. Behavioral Ecology and Sociobiology 1993; 33:225-231.
82. Johnston RE, Muller-Schwartz D, Sorensen PW. Advances in Chemical Signals in Vertebrates: Klewer/ Plenum, New York; 1999.
83. Lynch CB. Inbreeding effects upon animals derived from a wild population of Mus musculus. Evolution 1977; 31:526-537.
84. Connor JL, Belucci MJ. Natural selection resisting inbreeding depression in captive wild house mice (Mus musculus). Evolution 1979; 33:929-940.
85. Meagher S, Penn DJ, Potts WK. Male-male competition magnifies inbreeding depression in wild house mice. Proc Natl Acad Sci USA 2000; 97:3324-3329.
86. Ilmonen P, Penn DJ, Damjanovich K et al. Experimental infection magnifies inbreeding depression in house mice. J Evol Biol 2008; 21:834-841.
87. Neff BD, Pitcher TE. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. Mol Ecol 2005; 14:19-38.
88. Wedekind C, Chapuisat M, Macas E et al. Non-random fertilization in mice correlates with MHC and something else. Heredity 1996; 77:400-409.
89. Rülicke T, Chapuisat M, Homberger FR et al. MHC-genotype of progeny influenced by parental infection. Proc R Soc Lond B 1998; 265:711-716.
90. Doherty PC, Zinkernagel RM. Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. Nature 1975; 256:50-52.
91. Hughes AL, Nei M. Pattern of nucleotide substitution at major histocompatibity complex class I loci reveals overdominant selection. Nature 1988; 335:167-170.
92. Hill AV, Allsopp CE, Kwiatkowski D et al. Common west African HLA antigens are associated with protection from severe malaria. Nature 1991; 352:595-600.
93. Thursz MR, Thomas HC, Greenwood BM et al. Heterozygote advantage for HLA class-II type in hepatitis B virus infection. Nat Genet 1997; 17:11-12.
94. Carrington M, Nelson GW, Martin MP et al. HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science 1999; 283:1748-1752.
95. Arkush KD, Giese AR, Mendonca HL et al. Resistance to three pathogens in the endangered winter-run chinook salmon (Oncorhynchus tshawytscha): effects of inbreeding and major histocompatibility complex genotypes. Canadian Journal of Fisheries and Aquatic Science 2002; 59:966-975.
96. Penn DJ, Damjanovich K, Potts WK. MHC heterozygosity confers a selective advantage against multiple-strain infections. Proc Natl Acad Sci USA 2002; 99:11260-11264.
97. Froeschke G, Sommer S. MHC class II DRB variability and parasite load in the striped mouse (Rhabdomys pumilio) in the Southern Kalahari. Mol Biol Evol 2005; 22:1254-1259.
98. Hraber P, Kuiken C, Yusim K. Evidence for human leukocyte antigen heterozygote advantage against hepatitis C virus infection. Hepatology 2007; 46:1713-1721.

99. McClelland EE, Penn DJ, Potts WK. Major Histocompatibility Complex Heterozygote Superiority during Coinfection. Infect Immun 2003; 71:2079-2086.
100. Kekalainen J, Vallunen JA, Primmer CR et al. Signals of major histocompatibility complex overdominance in a wild salmonid population. Proc Biol Sci 2009; 276:3133-3140.
101. Oliver MK, Telfer S, Piertney SB. Major histocompatibility complex (MHC) heterozygote superiority to natural multi-parasite infections in the water vole (Arvicola terrestris). Proc Biol Sci 2009; 276:1119-1128.
102. Penn D, Potts W. The evolution of mating preferences and major histocompatibility genes. Am Nat 1999; 153:145-164.
103. Bertoletti A, Sette A, Chisari FV et al. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T-cells [see comments]. Nature 1994; 369:407-410.
104. Moskophidis D, Zinkernagel RM. Immunobiology of cytotoxic T-cell escape mutants of lymphocytic choriiomeningitis virus. J Viriol 1995; 69:2187-2193.
105. McMichael AJ, Phillips RE. Escape of human immunodeficiency virus from immune control. Annu Rev Immunol 1997; 15:271-296.
106. Jeffery KJ, Usuku K, Hall SE et al. HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy. Proc Natl Acad Sci USA 1999; 96:3848-3853.
107. Allen TM, O'Connor DH, Jing P et al. Tat-specific cytotoxic T-lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature 2000; 407:386-390.
108. Bowen DG, Walker CM. Mutational escape from CD8+ T-cell immunity: HCV evolution, from chimpanzees to man. J Exp Med 2005; 201:1709-1714.
109. Furukawa Y, Tara M, Izumo S et al. HTLV-I viral escape and host genetic changes in the development of adult T-cell leukemia. Int J Cancer 2006; 118:381-387.
110. Goulder PJ, Watkins DI. HIV and SIV CTL escape: implications for vaccine design. Nat Rev Immunol 2004; 4:630-640.
111. McClelland EE, Granger DL, Potts WK. MHC-dependent susceptibility to Cryptococcus neoformans in mice. Infection and Immunity 2003; 71:4815-4817.
112. Buseyne F, Janvier G, Teglas JP et al. Impact of heterozygosity for the chemokine receptor CCR5 32-bp-deleted allele on plasma virus load and CD4 T-lymphocytes in perinatally human immunodeficiency virus-infected children at 8 years of age. J Infect Dis 1998; 178:1019-1023.
113. Misrahi M, Teglas JP, N'Go N et al. CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. French Pediatric HIV Infection Study Group. Jama 1998; 279:277-280.
114. MacDonald KS, Embree J, Njenga S et al. Mother-child class I HLA concordance increases perinatal human immunodeficiency virus type 1 transmission. J Infect Dis 1998; 177:551-556.
115. Polycarpou A, Ntais C, Korber BT et al. Association between maternal and infant class I and II HLA alleles and of their concordance with the risk of perinatal HIV type 1 transmission. AIDS Res Hum Retroviruses 2002; 18:741-746.
116. Woelfing B, Traulsen A, Milinski M et al. Does intra-individual major histocompatibility complex diversity keep a golden mean? Philos Trans R Soc Lond B Biol Sci 2009; 364:117-128.
117. Wegner KM, Kalbe M, Kurtz J et al. Parasite selection for immunogenetic optimality. Science 2003; 301:1343.
118. Kalbe M, Eizaguirre C, Dankert I et al. Lifetime reproductive success is maximized with optimal major histocompatibility complex diversity. Proc Biol Sci 2009; 276:925-934.
119. Reusch TB, Haberli MA, Aeschlimann PB et al. Female sticklebacks count alleles in a strategy of sexual selection explaining MHC polymorphism. Nature 2001; 414:300-302.
120. Boehm T. Quality control in self/nonself discrimination. Cell 2006; 125:845-858.
121. Zahavi A. Mate selection-a selection for a handicap. J theor Biol 1975; 53:205-214.
122. Ditchkoff SS, Lochmiller RL, Masters RE et al. Major-histocompatibility-complex-associated variation in secondary sexual traits of white-tailed deer (Odocoileus virginianus): evidence for good-genes advertisement. Evolution 2001; 55:616-625.
123. Von-Schantz THWGGMGKP. MHC genotype and male ornamentation: genetic evidence for the Hamilton-Zuk model. Proc R Soc Lond B 1996; 263:265-271.
124. Ekblom R, Saether SA, Grahn M et al. Major histocompatibility complex variation and mate choice in a lekking bird, the great snipe (Gallinago media). Mol Ecol 2004; 13:3821-3828.
125. Milinski M, Griffiths SW, Reusch TB et al. Costly major histocompatibility complex signals produced only by reproductively active males, but not females, must be validated by a 'maleness signal' in three-spined sticklebacks. Proc Biol Sci 2009; 2010; 277:391-398.
126. Zuk M. The role of parasites in sexual selection: current evidence and future directions. Adv Study Behav 1992; 21:39-68.
127. Penn D, Potts WK. Chemical signals and parasite-mediated sexual selection. Trends Ecol Evol 1998; 13:391-396.

128. Kavaliers M, Choleris E, Agmo A et al. Olfactory-mediated parasite recognition and avoidance: linking genes to behavior. Horm Behav 2004; 46:272-283.
129. Moller AP, Saino N. Parasites, immunology of hosts and host sexual selection. J Parasitol 1994; 80:850-858.
130. Boehm T. Co-evolution of a primordial peptide-presentation system and cellular immunity. Nat Rev Immunol 2006; 6:79-84.
131. Han BW, Herrin BR, Cooper MD et al. Antigen recognition by variable lymphocyte receptors. Science 2008; 321:1834-1837.
132. Cooper MD, Alder MN. The evolution of adaptive immune systems. Cell 2006; 124:815-822.
133. Guo P, Hirano M, Herrin BR et al. Dual nature of the adaptive immune system in lampreys. Nature 2009; 459:796-801.
134. Scofield VL, Schlumpberger JM, West LA et al. Protochordate allorecognition is controlled by a Mhc-like gene system. Nature 1982; 295:499-502.
135. Grosberg RK, Hart MW. Mate selection and the evolution of highly polymorphic self/nonself recognition genes. Science 2000; 289:2111-2114.
136. De Tomaso AW, Nyholm SV, Palmeri KJ et al. Isolation and characterization of a protochordate histocompatibility locus. Nature 2005; 438:454-459.
137. De Tomaso AW, Weissman IL. Evolution of a protochordate allorecognition locus. Science 2004; 303:977.
138. Khalturin K, Bosch TC. Self/nonself discrimination at the basis of chordate evolution: limits on molecular conservation. Curr Opin Immunol 2007; 19:4-9.