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Report

Determination of Onset of Sexual Maturation and Mating Behavior by Melanocortin Receptor 4 Polymorphisms

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Summary

Polymorphisms in reproductive strategies are among the most extreme and complex in nature. A prominent example is male body size and the correlated reproductive strategies in some species of platyfish and swordtails of the genus *Xiphophorus*. This polymorphism is controlled by a single Mendelian locus (*P*) that determines the onset of sexual maturity of males. Because males cease growth after reaching puberty, this results in a marked size polymorphism. The different male size classes show pronounced behavioral differences (e.g., courtship versus sneak mating), and females prefer large over small males. We show that sequence polymorphisms of the melanocortin receptor 4 gene (*mc4r*) comprise both functional and non-signal-transducing versions and that variation in copy number of *mc4r* genes on the Y chromosome underlies the *P* locus polymorphism. Nonfunctional Y-linked *mc4r* copies in larger males act as dominant-negative mutations and delay the onset of puberty. Copy number variation, as a regulating mechanism, endows this system with extreme genetic flexibility that generates extreme variation in phenotype. Because Mc4r is critically involved in regulation of body weight and appetite, a novel link between the physiological system controlling energy balance and the regulation of reproduction becomes apparent.

Results

Understanding the genetic basis of polymorphisms, which can render individuals within a species seemingly more different than those between species [1], is a prerequisite to understand both their biological function and evolution. The winged-wingless dimorphism and its contingent migratory behaviors in insects have long been thought to be under control of a single gene [2]. Recently, a monogenic basis of a complex phenotype has been revealed for the rover/sitter polymorphism in *Drosophila melanogaster*. This is controlled by the *for* gene, which encodes a cGMP-dependent protein kinase [3], whereas homologous genes control foraging in honeybees [4] and the major/minor polymorphism in ants [5].

Polymorphisms in reproductive tactics within species are of special interest. In many cases, males exhibit extreme polymorphisms in morphologies, physiologies, behaviors, and life histories that promote reproduction by either conspicuously courting females or attempting to enforce copulations. There has been, however, little evidence that these characters have a strong (and simple) genetic underpinning [6].

A prominent example of polymorphism in reproductive strategy is male body size and the resulting behavioral diversity in two swordtail species of the genus *Xiphophorus*. Male *X. nigrensis* and *X. multilineatus* exhibit three main size classes: small, intermediate, and large [7, 8] (see Figure S1 available online). These body size differences are linked to a cascade of behavioral differences critical to the individual's evolutionary fitness. Large males defend territories that are visited by receptive females, whereas small males move among territories, readily expelled by larger males, while they search for females [9]. Larger males are better able than smaller males to maintain their locations in the high streamflow that characterizes some of these territories. Whereas large males court females in a ritualized fashion, small males do not court but perform "sneak matings" [10, 11]. Intermediate males court or sneak mate depending on their relative size compared to their opponents. Females clearly prefer large courting males, which thus have greater mating success in the wild [9, 12]. The estimated lifetime reproductive success of small versus large males, however, is equal, because the advantage of large male mating success is balanced by the advantage of faster-maturing small males being more likely to survive to maturity. Thus, the polymorphism is maintained by balancing selection [13].

Variation in body size in male *Xiphophorus* is linked to mechanisms triggering puberty. Because males cease to grow when they reach puberty, adult male body size is correlated with the time of sexual maturation: early-maturing males stay small, whereas late-maturing males grow large [14, 15]. Females, on the other hand, continue to grow throughout their lives [14]. Although most polymorphisms in reproductive strategies are facultative, being regulated by age, condition, or social environment, the *Xiphophorus* polymorphism is controlled by a single Mendelian locus that determines the onset of sexual maturity of males. This gene was called "pituitary locus" (*P*) because it was hypothesized to influence the hypothalamic-pituitary-gonadal (HPG) axis [8, 14, 16, 17].

The *P* locus was assigned to the sex chromosomes in the southern platyfish, *X. maculatus* [14]. A gene with several copies in the *P* region is the *melanocortin 4 receptor* (*mc4r*) gene [18]. Mc4r is a seven transmembrane G protein-coupled receptor. In mammals, Mc4r is expressed in the hypothalamus [19] and is involved in energy budget regulation through regulation of food intake [20]. A link to the hypothalamo-pituitary-gonadal axis is also likely [21], because Mc4r mediates leptin signaling, leading to gonadotropin releasing hormone

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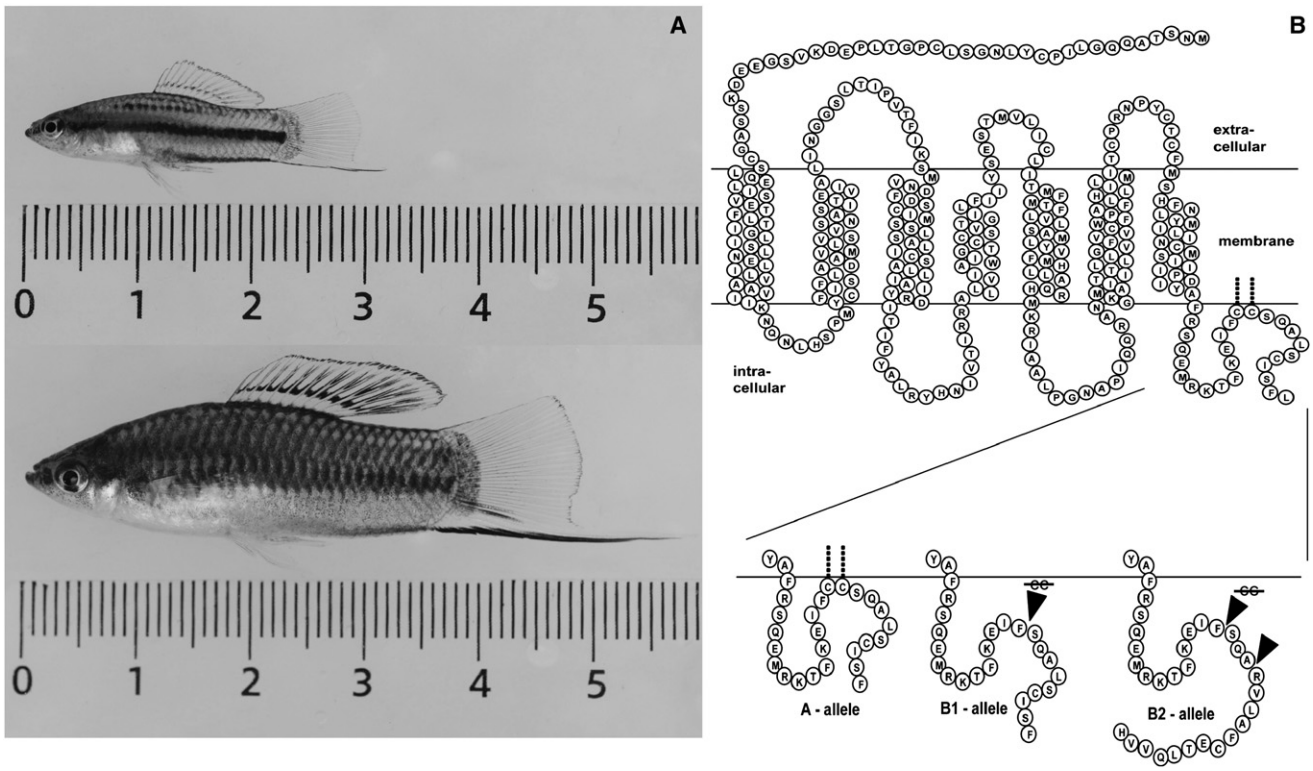

Figure 1. Male Size Variability and Mc4r Protein in *Xiphophorus* Fish

(A) Small (top) and large (bottom) males of *X. nigrensis*. See also Figure S1.

(B) Xiphophorus Mc4r protein and allele groups (A, B1, B2). Top: amino acid sequence and schematic structure of Mc4r (A allele group) from Xiphophorus. Bottom: carboxy-terminal tail of the Mc4R. A allele types represent the wild-type sequence. The B alleles are characterized by a six base deletion at the carboxyl terminus. Thus, all B allele types lack two cystein amino acid residues (~~CC~~). B2 alleles have an additional four base deletion that leads to a frameshift and elongated protein.

production [22], and is implicated in sexual function [23, 24]. Thus, we hypothesized that the *mc4r* cluster on the *X. maculatus* sex chromosome is the long-sought *P* locus.

Unfortunately, further analysis in this species is complicated by the fact that the *mc4r* cluster is present on the X and Y chromosomes, comprising up to 20 different copies even within an inbred strain of platyfish (C.S., J.-N.V., and C.H., unpublished data). Similarly, nine different alleles of the *P* gene have been identified. Thus, male platyfish show a continuous size range as a result of the large allele number [25]. To avoid these confounds, we focused our investigation on the above-mentioned *X. nigrensis* (Figure 1A) and *X. multilineatus*, which exhibit only three *P*-determined male size classes [12, 25, 26].

Copy Number Variation and Allele Classes of the Melanocortin 4 Receptor

To determine whether *mc4r* is involved in controlling the onset of puberty, we cloned the *mc4r* genes from *X. nigrensis* and *X. multilineatus* (Table S1). We found a large number of different alleles, indicating that *mc4r* is remarkably variable in these fish. Despite the high levels of allelic variability, however, the amino acid sequences of Mc4r in humans and *Xiphophorus* are highly conserved at functionally important positions [27].

The identified alleles constitute three major classes, A, B1, and B2, differing in the C-terminal intracellular region (Figure 1B). Although A alleles most closely resemble the functional *mc4r* of other vertebrates [28], B alleles lack two cysteine residues in the C-terminal region. In addition, B2 alleles have another four base deletion, leading to a frameshift and consequently an elongated carboxyl terminus.

A alleles were present in all animals investigated. Females had one or two different A alleles, whereas males had a maximum of four. Interestingly, very few different A alleles were found at all. We identified a total of 12 very similar A alleles within all the cloned and sequenced individuals (n = 16). One of the alleles was present at high frequency and was found in almost all individuals (evenness A alleles = 0.19).

Remarkably, B alleles were found (Table S1) only in males. They were present in all large and intermediate males, but in only some small males. Other small males lacked B alleles but are homozygous for A alleles, as are the females. Large males had up to 11 different B alleles. There was substantially greater variation in number of B versus A alleles (B1 = 19, B2 = 8; allelic richness: A = 12, B = 22). Also, in contrast to A alleles, no individual B allele was found to be abundantly present; instead, they were more evenly distributed among individuals (evenness: B1 = 0.40, B2 = 0.63). Intriguingly, B allele variability (27 alleles in 16 individuals) was found to be as high as major histocompatibility complex variability reported for poeciliid fish [29, 30].

In *X. nigrensis* and *X. multilineatus*, sex determination usually follows an XY pattern. An exception is XX males, which arise from the action of an autosomal modifier [31]. XX males are restricted to the small size class and are phenotypically indistinguishable from small XY males. Small males that were homozygous for A alleles were XX males (data not shown).

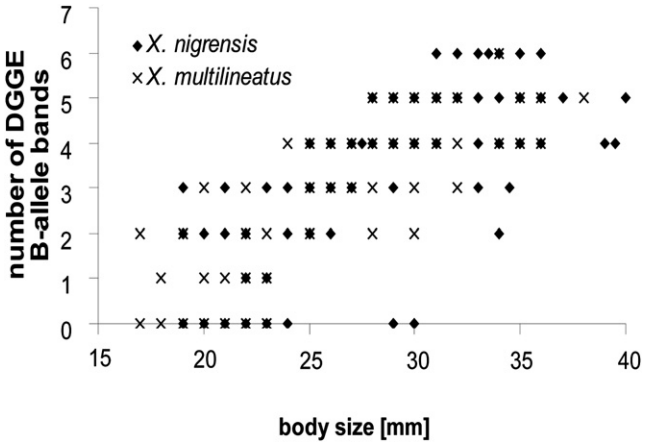

Figure 2. Correlation of Male Body Size and *mc4r* B Allele Copy Number, Determined by Differential Gradient Gel Electrophoresis

The x axis shows the individual body size, and the y axis shows the individual copy number of *mc4r* B alleles in heterozygous *Xiphophorus nigrensis* (diamonds) and *X. multilineatus* (crosses), determined by denaturing gradient gel electrophoresis (DGGE) analysis. Pearson correlation *X. nigrensis*: all males (n = 103) R = 0.73, R^2 = 0.53, p < 0.01; only heterozygous males (n = 87) R = 0.67, R^2 = 0.49, p < 0.01; *X. multilineatus*: all males (n = 76) R = 0.79, R^2 = 0.62, p < 0.01; only heterozygous males (n = 64) R = 0.74, R^2 = 0.55, p < 0.01. See also Figure S2 and Table S1.

This links the *mc4r* allele classes to the genetic sex-determining system, with A alleles containing X chromosomes and B alleles always assigned to the Y chromosome (χ^2 = 15.357, df = 1, p < 0.001).

Correlation of Male Body Size and *mc4r* Allele Copy Number

Individual size was not linked to a specific allele group or a specific allele within a group. Instead, Southern blot analysis revealed that male size seemed to be correlated with B allele number (Figure S2). This finding was confirmed by cloning and sequencing *mc4r* genes from individuals of all size classes (Table S1) and by denaturing gradient gel electrophoresis (DGGE) analysis that included a total number of 277 individuals (*X. multilineatus*: 76 males, 25 females; *X. nigrensis*: 103 males, 73 females). Although females never showed any B alleles, male body size was correlated with B allele copy number (Figure 2). More than half of the variation in adult male size is explained by B allele copy number (r^2 = 0.50 – 0.60). Although other minor genetic and environmental factors might influence male size, the number of Y-chromosomal *mc4r* B alleles seems to be of predominant importance.

Higher Expression of *mc4r* in Large and Intermediate Males

Expression of *mc4r* in *X. nigrensis* and *X. multilineatus* was found preferentially in the brain (Figure 3A). Intriguingly large and intermediate males had 10- to 15-fold higher levels than females (Figure 3B). Small XX males had low expression, similar to females. Small XY males displayed higher transcript levels than XX males but lower transcript levels than intermediate and large males. The high level in large and intermediate males was reached prior to puberty, persisted during sexual maturation, and was maintained during the entire lifetime (Figure 3 and data not shown). Allele-specific real-time quantitative polymerase chain reaction revealed that the higher expression in larger males is contributed by the Y-linked B alleles (Figure 3C). The small XX males showed only expression of A alleles and at similar levels as females. Consistently, the small XY males had lower levels of B allele expression than the intermediate and large males.

Signal Transduction Capacity versus Nonfunctionality of A and B Alleles

The most obvious difference between the A and B alleles is the presence or absence of a dicysteine motif in the C-terminal region of the protein (Figure 1B). This CC motif is highly conserved in vertebrate *mc4r* genes [32]. Such a motif is frequently found to determine the end of helix VIII of G protein-coupled receptors and is often lipid-modified by palmitoylation [32–34]. The CC motif is thought to stabilize the additional cytoplasmic helix VIII, probably by anchoring the protein tail to the membrane. This helix appears to play a critical role in receptor activation; thus, mutations of the CC motif could lead to disruption of receptor signaling, internalization, and desensitization [34]. To test the hypothesis, we established HEK cells that stably express the A or B forms of *X. nigrensis* Mc4rs. These cell lines were monitored for receptor-dependent second messenger production and reporter gene expression in response to stimulation with melanocortin. We found that receptors encoded by both types of B alleles could not be stimulated, whereas when stimulated by the ligand, receptors exhibited both higher cAMP production in membrane preparations (Figure 4) and reporter gene activation (Figure S3). This indicates that A alleles are functional, whereas the two B allele classes encode receptors that are incapable of transmitting a receptor signal. When functional and nonfunctional alleles were stably coexpressed in the same cell, higher levels of B allele expression decreased the reporter gene expression level of A alleles (Figure 4; Figure S3).

Discussion

The basis for a crucial role of *mc4r* in puberty onset in *Xiphophorus* fish is the presence of multiple copies that encode different isoforms. We speculate that non-signal-transducing Mc4r variants (encoded by B alleles) reduce the formation of functional Mc4r dimers or sequester the MSH ligand and therefore delay the onset of puberty. The number of B alleles in the genome is clearly correlated to adult male body size. A possible explanation is that the signal from functional Mc4rs needs to reach a certain level before the HPG axis is upregulated and sexual maturation is initiated. The threshold is reached early in females and in the small males homozygous for the functional A alleles. B alleles would delay the onset of puberty by diminishing the signal from the functional A alleles. The more B alleles present, the longer it would take until the threshold is reached. The longer it takes for a male to start puberty, the larger it will be as an adult. Interestingly, a naturally occurring nonfunctional truncated receptor variant has also been described for the G protein-coupled ghrelin receptor, with respect to downstream signaling. Like the B alleles forms of the *Xiphophorus* Mc4r, the truncated ghrelin receptor is expressed and diminishes the signaling of the wild-type receptor by acting as a dominant-negative mutant [35].

Although B forms of the melanocortin 4 receptor are nonfunctional as signal transducers, there appears to be selection on the gene to produce a structurally intact protein. In no case did we find stop codons or mutations that would compromise the production of Mc4r proteins. We interpret this as indicative that either the protein itself is involved

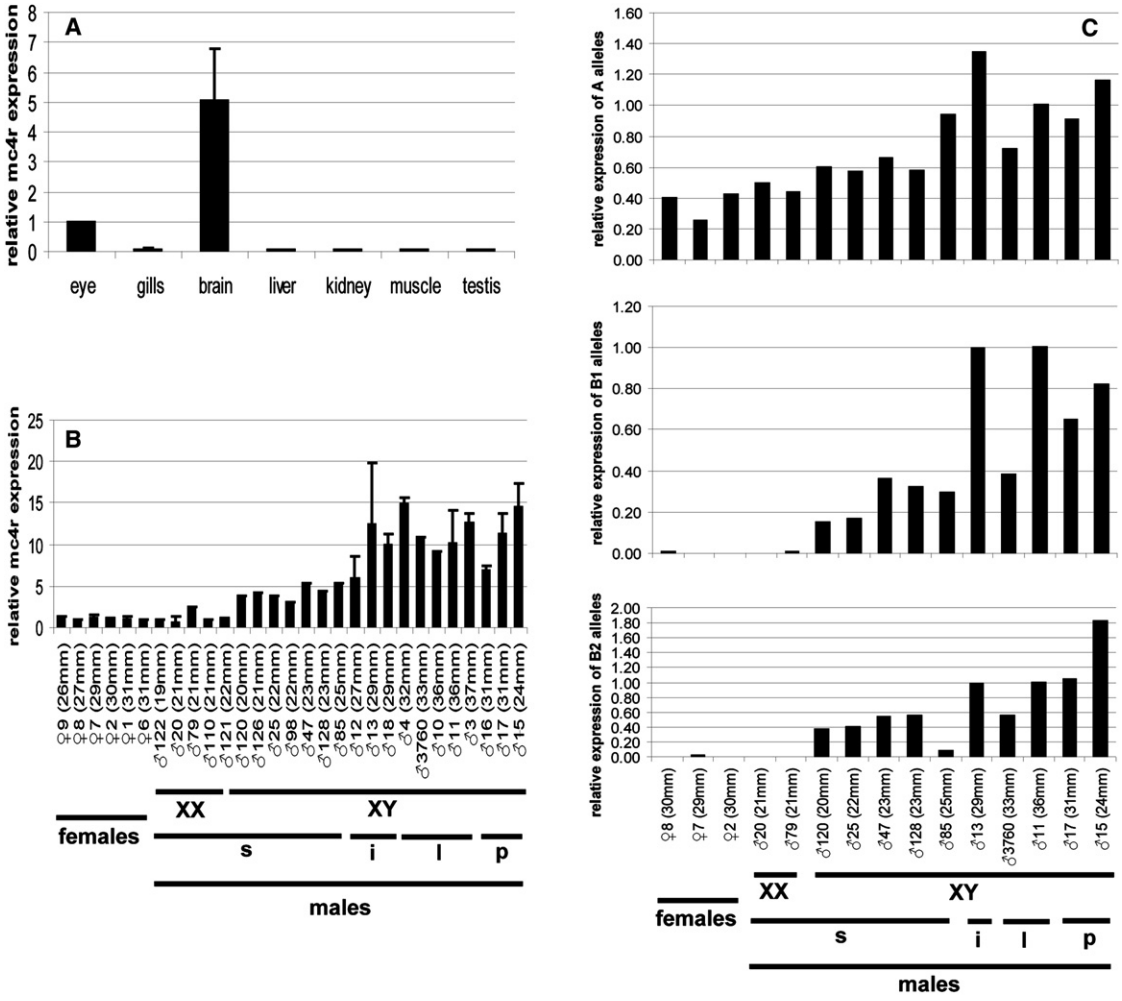

Figure 3. *mc4r* mRNA Expression in *Xiphophorus nigrensis*

(A) Expression of *mc4r* in different tissues of an individual male, using primers for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) that amplify all allele types. On the x axis the different tissue types are shown, and on the y axis the relative expression of *mc4r* is given. *mc4r* expression was standardized to the expression in the eye (expression level = 1). Given are mean values and standard deviation.

(B) Total *mc4r* expression in brains of different individuals. *mc4r* expression determined as in (A) was standardized to female #8 (expression = 1). The following abbreviations are used: p, prepubertal; s, small; i, intermediate; l, large. Given are mean values and standard deviation.

(C) Expression of A, B1, and B2 alleles in brains of male and female *X. nigrensis*, determined by qRT-PCR using allele-specific primers. Given are the relative expression (male #11 expression = 1) of the A, B1, and B2 alleles in females and different males. The following abbreviations are used: p, prepubertal; s, small; i, intermediate; l, large.

in the molecular mechanism, e.g., as a dominant-negative version implicated in downregulating the signal transmitted through the A allele-encoded receptor, or that the B allele gene products still have a very low biochemical activity that could not be measured but is sufficient and necessary to transmit a basal level signal in males.

Our findings that polymorphisms and copy number variation of the *mc4r* gene are linked to the onset of puberty in male *Xiphophorus* fish are consistent with a connection between energy balance and reproduction. In general, the onset of sexual maturation is more closely associated with body growth than with chronological age, because inadequate nutrition retards growth and delays sexual maturation [36, 37]. The hormonal system conveying metabolic information to the brain centers that govern reproductive function is highly complex and has yet to be fully explored. Leptin, a hormone derived from adipose cells, appears to play a key role in mediating this connection through a putative leptin-kisspeptin-gonadotropin-releasing hormone pathway [38, 39]. In the hypothalamus, the regulation of energy balance critically involves the Mc4r system [40]. The melanocortins have been suggested to participate in the downstream events of leptin signaling events [41]. Thus, changes in the Mc4r system could be expected to have an effect on the onset of puberty.

In *Xiphophorus*, male mating behavior is correlated with adult size, so the polymorphism in *mc4r* genotype also results in a behavioral polymorphism. Female preference for large males likely exerts selection on *mc4r* B allele copy number. An interaction between female mate choice and male genetic polymorphism has been proposed for other animal groups [42–44]. In no other system, however, is the link between reproductive success in the wild, female mating preferences, and genes that contribute to phenotypic variation subject to sexual selection as obvious as it is here in *Xiphophorus*. In

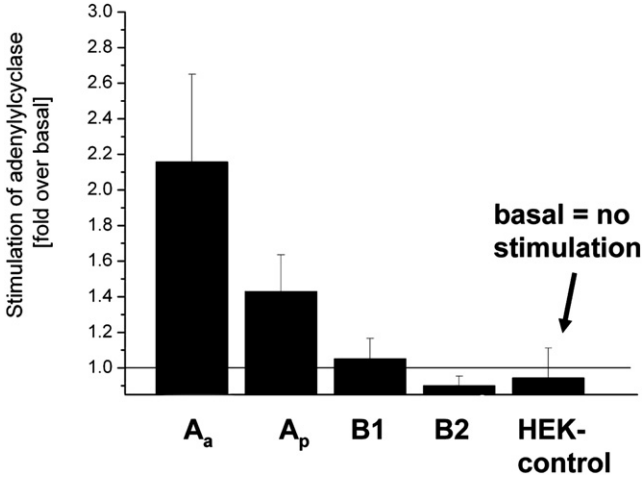

Figure 4. Cyclic AMP-Producing Activity of A and B Allele-Encoded Mc4rs

Stimulation assay of adenylylcyclase activity with the melanocortin receptor agonist NDP-αMSH from membrane preparations for two different A alleles, one B1 allele, and one B2 allele in comparison with wild-type HEK cells. Given are mean values and standard deviations. See also Figure S3.

addition, the dosage-dependent effect of *mc4r* copy number makes the system extremely flexible. Copy number variation, which became a recent focus in human genetics, might be a general mechanism regulating complex phenotypes.

Experimental Procedures

See Supplemental Experimental Procedures.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at doi:10.1016/j.cub.2010.08.029.

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Supplemental Information

Determination of Onset of Sexual Maturation and Mating Behavior by Melanocortin Receptor 4 Polymorphisms

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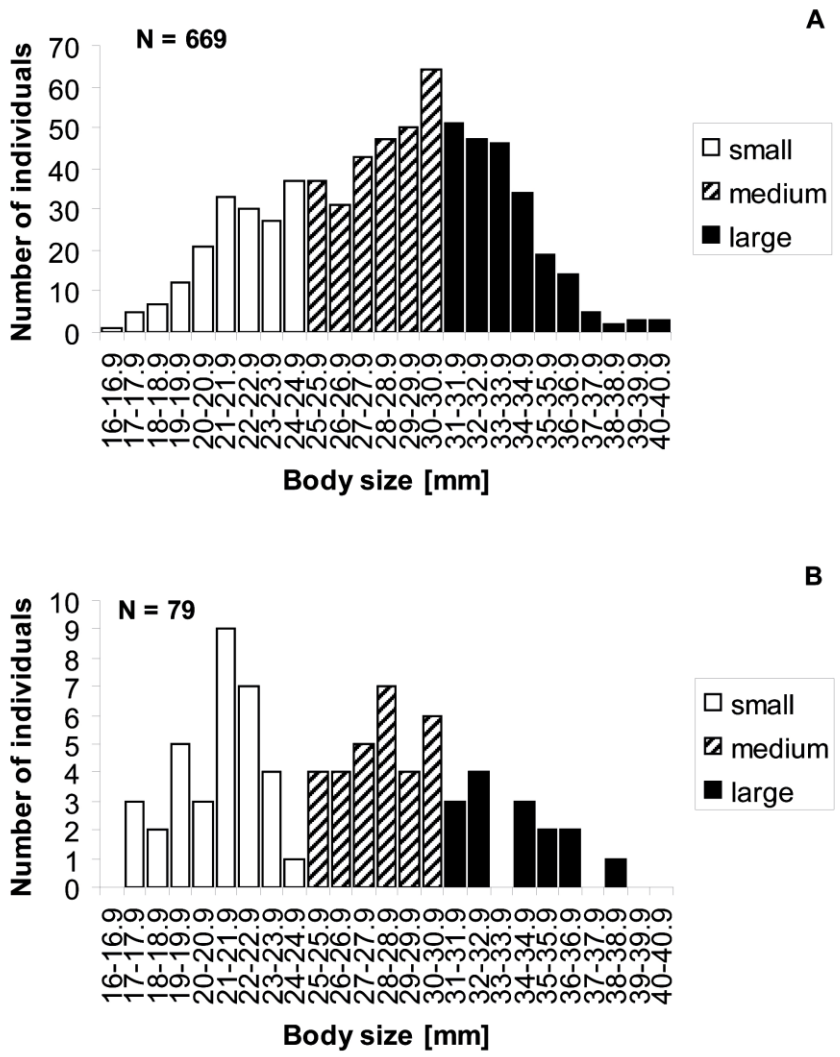

Figure S1 (Related to Figure 1A). Distribution of Male Body Sizes in a Natural Population of A) X*iphophorus nigrensis* (Rio Choy) and B) *Xiphophorus multilineatus* (Rio Coy)

The x-axis shows the body size [mm] of animals caught in the field, the y-axis the frequency of the size class. Size classes overlap. The ranges have been set according to crossing experiments and behavioral studies (<25mm = small; 25-31mm = intermediate, > 31mm = large)[1] and are depicted in white (small), black (large) or as a striped pattern (intermediate). Sample sizes vary due to differing sampling efforts (*X. nigrensis* N = 669, for *X. multilineatus* N = 79).

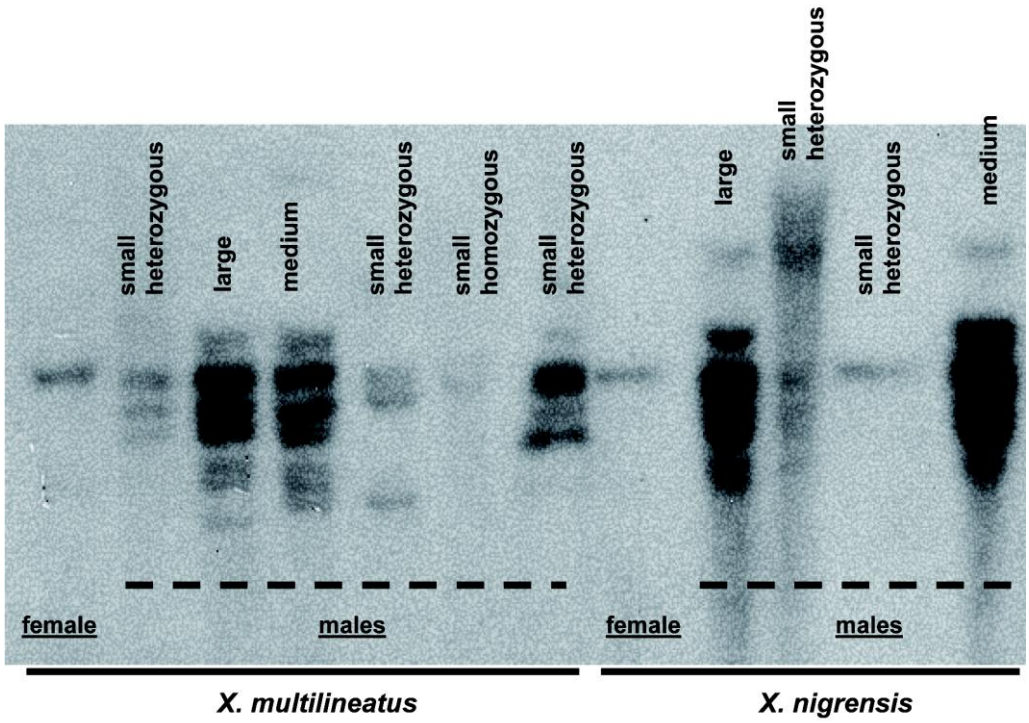

Figure S2 (Related to Figure 2). Southern Blot Analysis of *mc4r* Copy Number in *Xiphophorus multilineatus* and *X. nigrensis* of Different Body Size
Small homozygous males show the same allelic pattern as females. Small heterozygous males have less gene copies than large and medium size males.

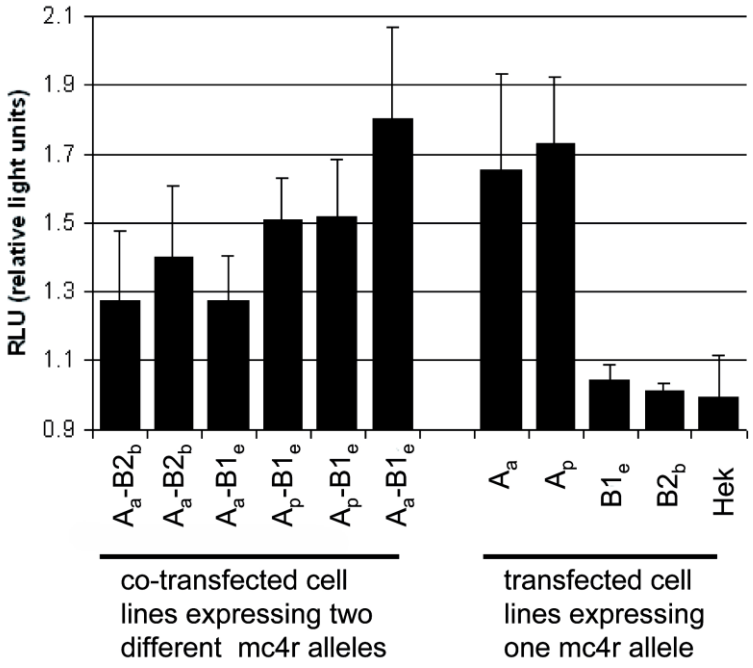

Figure S3 (Related to Figure 4). Allelic Expression and Mc4r Activity in Different Cell Lines

Reporter gene expression in stable cell lines after receptor stimulation with NDP-αMSH. Given are mean values and standard deviations for cell lines expressing a single *mc4r* allele or two *mc4r* alleles from different groups. Cells stably expressing a single allele type show clear activity only for the A alleles while B alleles are non-functional. A allele cell lines that co-express B-alleles at least to the same level or above have a reduced reporter gene activity. Ratio of A:B allele expression, determined by allele specific RT-PCR, from left to right A_a: $B2_b$ 1:1.7, A_a:$B2_b$ 1:2.3, A_a:$B1_e$ 1:3.3, A_p:$B1_e$ 1:3.9, A_p:$B1_e$ 1:0.8, A_a:$B1_e$ 1:0.5. HEK, non-transfected control.

Table S1 (Related to Figure 2). Body Size and *mc4r* Copy Number Determined from Cloned PCR Products of *Xiphophorus nigrensis* and *X. multilineatus*

Ind #	Species	Sex	Body Length [mm]	# Clones'	# of A	# of B1	# of B2	total # of B
3119	Xm	f	22	10	1	0	0	0
3120	Xm	f	23	7	1	0	0	0
903	Xm	m	22	11	2	0	0	0
905	Xm	m	19	26	1	4	1	5
902	Xm	m	21	17	1	1	1	2
896	Xm	m	21	33	1	4	0	4
908	Xm	m	26	25	1	2	4	6
900	Xm	m	29	17	2	4	2	6
898	Xm	m	34	46	1	8	2	10
1151	Xm	m	35	29	4	8	3	11
886	Xn	m	20	7	1	0	0	0
892	Xn	m	19	19	3	3	3	6
878	Xn	m	23	13	2	2	2	4
891	Xn	m	25	28	3	7	1	8
877	Xn	m	33	27	1	7	3	10
874	Xn	m	35	17	1	6	3	9

(Xm – *X. multilineatus*, Xn – *X. nigrensis*; f – female, m – male; #-clones – number of clones analyzed)

' a discrepancy between the number of clones analyzed and number of A and B alleles identified is due to single sequences not being taken into account to avoid false allele variability estimates due to methodological problems (template switch).

Table S2. Primer Sequences

Name	Sequence (5' – 3')
PCR Amplification and DGGE Analysis	
F10	CCTTCCACCGTTTCTCCT
F1	GAACTCCACGGCTCAGCAAG
F680	CACGTTCATC AAAAGCATGG
F929	CACGGGGGGCACCATGTTCTTCACCATGC
R5	GACATTTCAGGCTCTTCATCC
AR1	GAGAGCCTGTGAGCAGCAGA
GC-clamp	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG
Realtime-PCR	
Xmn_MC4R_f2	CAGCCAGGAGATGAGGAAGA
Xmn_MC4R_r1	GCACTGCACACGGATTACAG
Taqman- Primer + Probes	
FW-ELF	CAAAAGGAAGTGAGCACCTACATC
RV-ELF	CCAAGGCATCCAGGAGAGTG
PR-ELF	[6FAM]CCTGCCGCTGTTGCCTTCGTCC[TAM]
FW	TGCTTCATGTCGCACTTCAAC
PR-B1	[6FAM]a+Gag+Agcctgtga+Gaa+Ga[BHQ1]
PR-B2	[6FAM]agct+Aata+Cacga+Gc+Ctg [BHQ1]
PR-A	[6FAM]cctgtg+Agc+Ag+Cagaa [TAM]
RV	GCACTGCACACGGATTACAG

Supplemental Experimental Procedures

Animal Sampling and DNA Extraction

Animals were sampled in their natural habitats in Mexico in 1987 and 1988 (size measurements) and 2010 or came from laboratory strains bred from animals obtained in the same field trip. Field caught *Xiphophorus nigrensis* were collected from the nacimiento of the Río Choy (N 21° 59.264' W 98° 53.106'), and *X. multilineatus* from the Río Coy (N 21° 45.096' W 98° 57.445'). Note that *X. multilineatus* in the earlier literature was designated as the "Rio Coy population" of *X. nigrensis*, and was only recently recognized as a separate species [2]. Individuals were captured using seine nets. Standard and total length of all males was recorded. To obtain genomic DNA animals were either fin clipped and released immediately after biopsy or they were killed by cervical dislocation. Pooled organs from individual animals (brain, gills, liver, spleen, kidney) were immediately lysed in a sample storage buffer [3]. Tissues were stored in this buffer until further analyses and processed as described [3, 4].

Cloning and Sequencing

Mc4r alleles were cloned and sequenced in 10 *Xiphophorus multilineatus* and 6 *X. nigrensis*. PCR amplification of the *mc4r* genes was performed using the primers F10 or F1 and R5 (Table S2), which amplify the full open reading frame of the gene. PCRs were performed in a 25µl volume using 2.5µl DNA (50-100ng/µl), 2.5µl buffer (1x), 4µl dNTP (10pmol/µl), 0.5µl of each primer (10pmol/µl each), 1.25µl DMSO and 0.25µl Taq. The PCR was run with an initial denaturing time of 5min at 94°C, 40 cycles of 94°C 45sec, 55°C 45sec, 72°C 60sec and a final elongation step of 5min at 72°C. PCR products were cloned into a pCRII TOPO vector and transformed in *Escherichia coli* competent cells (recA- DH5α strain K12 derivate) following the provider's (Invitrogen Ltd., Karlsruhe, Germany) recommendations. A minimum of 7 positive clones per individual was sequenced using the plasmid integrated M13 forward and reverse primers. Sequence processing and alignments were performed with BioEdit. To minimize PCR induced errors (template switch) we used the minimum number of cycles for PCR amplification and a Taq mixture that included 10% proofreading Taq. In addition, all sequences were visually inspected and each determined *mc4r* allele sequence was confirmed from a minimum of two clones that came from two independent PCR and cloning reactions.

Southern Blot Analysis

Total genomic DNA was digested with EcoRI, separated on 0.8% agarose gel, transferred to nitrocellulose membrane and hybridized with the X. nigrensis mc4r A-allele probe.

DGGE Analyses

To allow population level screening for *mc4r* copy numbers we used a denaturing gel gradient (DGGE) approach (DGene system, BioRad, Hercules, CA, USA). PCR products of clones with known DNA sequences as well as from sequenced individuals were used to establish maximum resolution in the DGGE-gels. To determine the separation threshold of these conditions we artificially mixed the two most similar alleles (A_a and A_p = 5 differences in 987 base pair

sequence). 30µl of PCR product from individual fish were loaded onto the gel and copy number was recorded for each analyzed individual. Primers F929 and R5 were used to determine total *mc4r* copy number, primers F929 and AR1 were used to determine the number of A-alleles present in each individual (details on the primers sequences are given in the supplemental material Table S2). Optimal separating conditions were: 6.5% polyacrylamide solution (37.5:1 ratio acrylamid/bisacrylamid), 30-50% urea gradient, run temperature 60°C, runtime 20h, current 100 Volt.

Expression Analysis

Total RNA was prepared from individual organs of freshly killed laboratory animals or field collection samples stored in RNAlater (Sigma, Munich, Germany) and extracted using Total RNA Isolation Reagent (ABgene, Hamburg, and Peqlab, Erlangen, Germany) according to the supplier's recommendation. After DNase treatment, reverse transcription was performed from total RNA using RevertAid First Strand Synthesis kit (Fermentas, St. Leon-Rot, Germany) and random hexamer primers according to the manufacturer's instructions. cDNA was used for realtime PCR (for primer sequences see Supplementary Material Table S2) using SYBR Green reagent, and amplification was monitored with an iQ5-Cycler (Bio-Rad, Munich, Germany). All results reported here are generally averages of at least 2 independent reverse transcription (RT) reactions and 2 PCR experiments from each such reaction. For quantification, data were analyzed by the delta Ct method [5], and normalized to the housekeeping gene *ef1a* mRNA.
For determining total *mc4*r expression levels 50ng cDNA from 2µg total RNA was used for RT-PCR (for primer sequences see Supplementary Material Table S2, reaction conditions 95°C for 3min; 40 cycles at 95°C for 30sec and 60°C for 30sec).
Primers and specific TaqMan probes for allele specific RT-PCR were designed based on the diagnostic differences in the carboxy-terminal part of the A, B1 and B2 alleles (for primer sequences see Supplementary Material Table S2). Reactions (100ng cDNA from 2µg total RNA in JumpStart Taq Ready Mix) were conducted on an iCycler iQ5 Real Time PCR equipment (94°C for 2min; 40 cycles at 94°C for 15sec and 60°C for 1min), visualized using iCycler iQ Real-Time Detection System Software. Duplicates of each sample were used both for standard curve generation and during experimental assays.
For negative control RNA (not reversely transcribed) was used in the PCR reaction. Data are presented as mean ± standard deviation.

Biochemical Analyses of Receptor Function

HEK 293T cells were stably transfected with constructs expressing different *mc4r* alleles under the control of the CMV promoter. The corresponding genomic fragments containing the full open reading frames were amplified with primers (hf1: 5'ATTGCTaagcttACCCATTCAGGACTTGCTGT3' and xr1: 5' TTCGTGctcgagGCACTGCACACGGATTACAG3'), cut with *Hin*dIII and *Xho*I and inserted into the corresponding sites of pcDNA3 or pcDNA 3.1/Zeo(+). Stimulation of cells and adenylyl cyclase assays (determination of production of [^{32}P]-cAMP) from membrane preparations were performed as described in Volff et al. [6]. NPD-αMSH (Sigma) was used as ligand and forskolin for activation control. For measuring the transcriptional activating activity of *mc4r* alleles a reporter gene luciferase assay was performed essentially as described in Sturm et al [7] except

that 500ng of reporter gene plasmid and 200ng of the control plasmid were used for transfection and that NDP-αMSH was added only 22h after transfection. Receptor expressing cell lines were transiently transfected with pSgIICRE^{4}luc [8] using polyethylenimine [9]. For quantification the pRL-CMV vector expressing renilla luciferase (Promega) was cotransfected. For positive control the catalytic subunit of PKA fused to nuclear localization signals was coexpressed from plasmid pCMV-Flag-NLS-Cα [8]. Luciferase was determined with the Dual Luciferase Reporter Assay System (Promega). All receptor assays were performed at least from three independent experiments.

Data and Statistical Analyses

Sequence alignments were done in BioEdit and Geneious using the ClustalW algorithm. RealtimePCR/TaqMan PCR results were standardized to brain=1, female#8=1 for total mc4r expression and male#11=1 for allele specific expression analysis. Correlations of body size and copy number were calculated using Pearson Product Moment correlation (Statistica). Fstat was used to determine allelic richness for the minimum number of 75 clones (number of A-alleles found in all analyzed clones).

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of this equilibration and the answer to the chicken-and-egg question of whether changes in nucleocytoplasmic transport cause nuclear entry of Rna1 or vice versa is unknown.

One possibility raised by Asakawa *et al.* [9] is that the physiological role of V-NEBD is to exclude certain proteins from the nucleus prior to spore formation. Consistent with this possibility, it is known that the volume of spore nuclei is less than that of growing cells [19]. V-NEBD, like NEBD, may also be a means of dissipating the Ran–GTP gradient across the NE and allowing the establishment of a chromosome-based Ran-GTP gradient known to be important for spindle formation in animal cells. However, V-NEBD is first observed at meiotic anaphase II, well after spindle assembly [8].

Deciphering the biological role of V-NEBD will be a challenging task but the fact that it is specific to meiosis II may provide important clues. Meiosis II-specific changes in spindle pole body anchoring in the NE could explain V-NEBD and its cell-cycle stage specificity but would not be consistent with the absence of an ultrastructural defect in the NE [9]. Regardless of the mechanism, it will be important to determine the functional relevance of V-NEBD at meiosis II by accomplishing the difficult task of creating conditions in which V-NEBD is prevented and then monitoring the consequences (see Figure 2C).

Open and closed mitosis are extremes of a process with many variations (Figure 1). The two new papers from Arai *et al.* [8] and Asakawa *et al.* [9] add a fascinating new twist by describing V-NEBD, which is structurally closed but functionally open and occurs only at meiosis II in *S. pombe,* an organism in which mitosis and meiosis I are structurally and functionally closed. The surprising observation of V-NEBD alone raises important questions about how and why it happens and why it happens only at meiosis II. In addition to furthering our understanding of yeast meiosis, the answers to these questions will bear on understanding the fundamental differences between open and closed mitosis and may provide insight into the evolution of nucleated cells.

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Reproductive Strategies: How Big Is Your Love?

In swordtails, gene copy number variation is associated with alternative male mating strategies, size and puberty. Though it is unclear how the different aspects are linked mechanistically, the nature of the gene, a melanocortin receptor, suggests avenues for future inquiry.

Florian Maderspacher

There are many paths to success, a truism illustrated in biology by the diversity of forms, lifestyles, and species. Even within a species, lifestyles can vary, as is exemplified in the alternative strategies males use for accomplishing their one purpose in life, mating. Male side-blotched lizards, for instance, come in three sizes — conveniently colour labelled orange, blue and yellow — that differ in mating behaviour [1]. Similarly, in a marine isopod, there are three types of male that vary considerably in their appearance yet are equally successful at reproducing [2]. Most spectacularly, perhaps, male Australian cuttlefish use their superior morphing skills to disguise temporarily as females and sneak their sperm into a mating couple [3]. Now, as they report in a recent issue of *Current Biology*, Kathrin Lampert, Manfred Schartl and colleagues [4]

Figure 1. Small male, what now?

Large (left) and small (right) male morphs of *Xiphophorus nigrensis*. The males differ in colouration and the elongated sword on the tail fin, for which females have a penchant. In the large male, the gonopodium is visible just behind the pelvic fins. Photo: Manfred Schartl.

have identified a gene associated with such alternative male strategies, in a fish, the Panuco swordtail (*Xiphophorus nigrensis*).

Fish are rather inventive when it comes to reproduction: there are monogamous, polygamous, promiscuous fish, some have parental care, others are careless egg-dispersers, a few don't have sex at all, others show sex-role reversal or full-on sex change. Likewise, male fish have found many paths to reproductive glory [5]. Males unusually invest heavily in sexual ornaments or elaborate courtship displays in order to score a mating. Contrasting these more traditional, or as they are fondly called, 'bourgeois' males, are the 'sneaker' males found in many species that quickly toss in their sperm while another couple is at it, or 'satellites' and 'cuckolders' that stand by and wait for an opportunity to covertly mate. Such sneaky males are essentially 'parasites' as they exploit the investment of the bourgeois male which is what gets the often choosy females receptive in the first place.

Obviously, these strategies must somehow pay off, and in some cases, like the bluegill sunfish, the 'proletarian' can be more successful than the bourgeois at siring more and better offspring [6]. These alternative male strategies can be genetically or environmentally induced, plastic or fixed. Yet, they have several features in common: the parasitic morph is usually significantly smaller and less ornate, often resembling females; the smaller males usually do not court; and, as parasitic males often interfere with another mating, they tend to invest heavily in making their sperm competitive [5].

In the fish that Schartl and colleagues [4] studied, *Xiphophorus nigrensis* (Figure 1), males come in three size classes — small, intermediate and large (the latter two often being lumped together) — which can differ in length by a factor of two [7]. The large males fit the 'bourgeois' description: they are decorated with the eponymous tail-sword, they are territorial and they engage in courtship displays. The small males, by contrast, have only a tiny sword and look more like females. Not surprisingly, females prefer to mate with the large ornate males. The small males do not court the females, but rather chase them and force them to copulate. (Unlike most fish, males of the Poeciliinae do not cast their sperm on laid eggs, but instead fertilise the female by virtue of a transformed anal fin, the gonopodium, and the females give birth to live young rather than laying eggs.)

The alternative male strategies, variations of which are found in several related species, have been intensely studied in *X. nigrensis* from both a genetic and an evolutionary point of view. The different male morphs are genetically determined, such that large males will almost always sire large male offspring and small males will have small sons. As first observed in the related platyfish (*X. maculatus*) [8], the genotype correlates with the activation of the gonadotropic part of the anterior pituitary gland that is — in fish, as in other vertebrates — responsible for triggering sexual maturation. The locus was thus called *pituitary* or *p*. As males of either genotype grow at the same rate, and stop as soon as they reach sexual maturity, the different sizes are simply a function of the timing of puberty: in small males, puberty starts early (at roughly 2.5 months), in large males late (at roughly 4 months). In platyfish and *X. nigrensis*, the *p* system was shown to be sex-linked such that the 'small', 'intermediate' and 'large' alleles of *p* are Y-linked, while the X chromosome only carries the 'small' allele (Figure 2).

This relatively straightforward genetic makeup allowed for a key evolutionary question to be addressed, namely, how do genotype frequencies change in response to the apparent sexual selection. In terms of getting to mate, the large and intermediate males are favoured by females and therefore the frequency of the small allele is decreasing from generation to generation [9]. But then how do the small males persist? It turns out that, when reproductive success is estimated over the entire lifetime, the two types of male — bourgeois and parasitic — may score roughly even [9]. This is because the smaller morph has two advantages: smaller males have more time to reproduce as they reach puberty earlier. In addition, they may also be better at evading predation as they're less conspicuous. As an aside, there is another reason why small males do not disappear over time. Unlike most fish, Panuco swordtails have a XY sex-determination system. However, as a result of the action of an autosomal modifier gene called *a*, some of the genotypically female XX individuals develop as small, swordless males when they are also *aa* homozygous [10].

To isolate the *p* locus, Schartl and colleagues [4] turned to a candidate gene that is present on the sex chromosomes, the melanocortin4 receptor *Mc4r*. They found a considerable amount of allelic variation at the locus, falling into two allele classes, termed 'A' and 'B', which differ in the cytoplasmic, carboxy-terminal end of the encoded transmembrane receptor: while the A alleles resemble the 'common' Mc4r versions of other vertebrates, all alleles in the B class show a conspicuous lack of two cysteines (along with various other small

changes). Biochemical analysis of A and B variants in a heterologous system shows that receptors of the B type fail to transduce a signal and, when co-expressed, can compromise the function of the A-types by either titrating away ligand or recruiting A-type receptors into non-functional complexes. The fact that even the non-signalling *Mc4r* alleles still code for a protein suggests that they in fact are maintained by evolution.

But is *Mc4r* more than just a candidate gene? If this was a fully-fledged model organism one would of course have tried to test Mc4r function directly, for example by transgenically expressing it in the various morphs and looking for a change in their reproductive type. But these kinds of experiments are not — yet? — feasible in *Xiphophorus* (and hindered by their internal fertilisation and live-bearing). But Schartl and colleagues [4] provide two main lines of evidence for association of *Mc4r* genotype with reproductive phenotype. First, following the sex-linked inheritance described for the *p* locus, A alleles are always X-linked, while B alleles are always Y-linked. In line with this, all animals, whether female or male, big or small, have at least one A allele (there are several A alleles, but only one has a high frequency). This indicates that at least one functional copy of *Mc4r* is required for viability. Likewise, as expected from the Y linkage, B alleles are found only in males.

But, as both large and small morphs both carry Y chromosomes with B type alleles of *Mc4r*, it is unlikely that there is a simple qualitative association of size with one or the other B-allele, all the more as the diversity of B-type alleles is extraordinarily high — in a sample of 16 fish there were 27 different alleles. Thus, rather than a qualitative association, the authors found — and this is the second line of evidence — a quantitative association: the larger the male, the more copies of B-alleles (up to 10) it carries. In quantitative genetic terms, 50–60% of the variation in male body size can be explained by B-allele copy number. This obviously leaves some 40% of the variation unaccounted for. These could be genetic or environmental factors as it is well known that despite the genetic determination the size of male morphs can fluctuate greatly with environmental conditions [11].

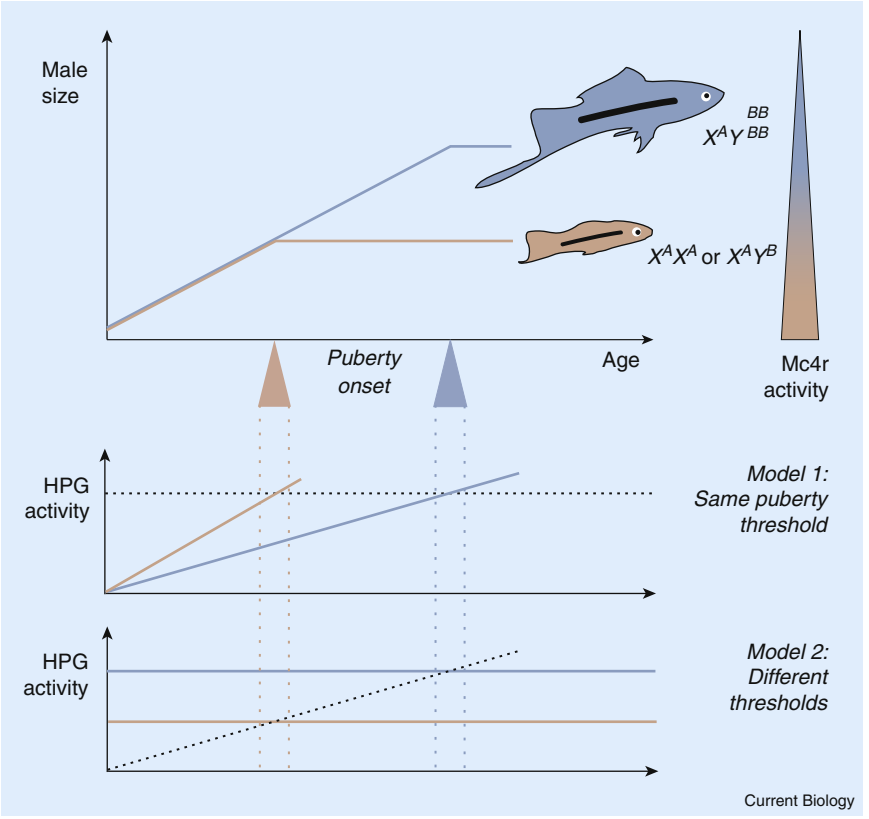

Figure 2. Sizing up: models for Mc4r function in determining male phenotype.
Top panel: Small (red) and large (blue) male morphs differ in the number of Y-chromosome-linked copies of a non-functional allele (B) of the *melanocortin 4 receptor* (*Mc4r*). High level Mc4r signalling causes early onset of puberty and hence arrested growth at small size, while lower Mc4r signalling allows for longer growth in large males. Onset of puberty is triggered via activation of the hypothalamus–pituitary–gonad (HPG) axis. Hypothetically (lower panels), different levels of Mc4r signalling might lead to attainment of a fixed threshold for HPG activation at different times (Model 1) or the HPG threshold levels themselves might differ depending on the level of Mc4r signalling (Model 2).

Variation in gene copy number, as observed for *Mc4r*, is becoming more and more appreciated as a significant contributor to genetic variation and phenotypic diversity, ever since the human genome was found to harbour nearly 40,000 sites of copy number variation, encompassing over 12% of its size [12,13]. Copy number variants have been found associated with complex human traits, such as autism or schizophrenia, or with HIV susceptibility [13]. Like the *Mc4r* story, so far these are only associations, but the explanatory appeal of copy number variants lies in the fact that they have the potential to influence gene dosage in a subtle way by altering a gene's expression levels — rather than altering its protein sequence. And indeed, in the large males *Mc4r* B alleles are expressed at a higher level. Such dosage effects may be particularly relevant for genes that have a pleiotropic function that would make the organism more vulnerable to their loss.

If one were to play devil's advocate, one could argue that the knowledge that *Mc4r* is the *p* gene does *per se* not add all that much to the well-understood evolutionary aspects of the reproductive strategies, the way they are selected, their costs and benefits. Nor does the molecular nature of *p* immediately explain how the phenotype comes about, neither in terms of its effects on puberty and growth nor on the mechanistically even less clear association with different kinds of behaviours. Obviously, the appeal of such a paper — apart from satisfying scientific curiosity — lies in the hope that the molecular identity of the gene identified can inform the biological mechanism underlying the trait. In that sense, *Mc4r* is a great candidate because its involvement

in the regulation of sexual maturity in male morphs was not entirely predictable, yet with some imagination such a role can be reconciled with Mc4r's known functions and is thus intriguing enough to stimulate further investigation [14]. For one, *Mc4r* acts in the hypothalamus, thus making a link to the hormonal hypothalamus–pituitary–gonad (HPG) axis that triggers sexual maturity in vertebrates at least plausible. In fact, in female rats Mc4r activity can affect expression of certain hormones of the HPG axis, such as LH and GnRH, but its role in males — aside from Mc4r stimulation promoting erection through direct action in the penis — is not well understood [14].

Mc4r is, of course, best known for its role in energy homeostasis where it mediates the effects of leptin in the hypothalamus. When an animal is hungry, leptin levels are low and, via lowered activity of POMC neurons, the activity of neurons secreting the Mc4r antagonist AgRP is increased, resulting in lowered Mc4r signalling. Low Mc4r activity means that food intake will be increased and energy expenditure decreased. These functions appear to be largely conserved across vertebrates, and it will be interesting to see if Mc4r affects puberty onset via direct action on the HPG axis or by some indirect means related to its function in energy homeostasis. There is some indication that bourgeois and parasitic morphs vary in their energy expenditure, though if and how this is linked to Mc4r function remains to be seen [15].

Perhaps the most fascinating question is how the traits that differ between the morphs — size, by way of puberty, and behaviour, especially mating and courtship — are connected. Are they both directly regulated by Mc4r, in a hard-wired fashion? Or is there some kind of plastic, feed-back mechanism, where for instance a smaller male adapts its behavioural strategy in response to the phenotype it has been dealt by its genetic makeup. Whether these questions will be studied in *Xiphophorus* or in other fish models, Mc4r offers a molecular handle on these processes and an especially relevant one at that, as it is the particular link in the system that evolution seems to have tweaked to endow male *X. nigrensis* with two different reproductive strategies. Hopefully, the small males will have a big future ahead of themselves.

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Ciliary Trafficking: CEP290 Guards a Gated Community

A recent study reveals that the large coiled-coil protein CEP290 is an integral component of the transition zone between the cell body and the cilium and functions as a gatekeeper to regulate trafficking of ciliary proteins.

Ewelina Betleja and Douglas G. Cole

Cilia and flagella are dedicated organelles with specialized functions that require protein and lipid compositions that differ considerably from the rest of the cell. These distinct compositions are maintained by a strict border policy that governs movement across the transition zone between the cell body and the cilium. The transition zone is a short region of the cilium that lies between the basal body and axonemal microtubules, where the basal body triplet microtubules transition into the axonemal doublets. Although the complex ultrastructure of the transition zone was beautifully documented in early studies by Ringo [1] and Gilula and Satir [2], its protein composition has largely remained elusive. Interest in this region, however, is increasing as researchers examine the transport of specific ciliary materials. Intraflagellar transport (IFT) particles powered by kinesin-2 and cytoplasmic dynein 1b, for example, must travel through the transition zone to enter and exit the organelle. Distinct from the transition zone are the transitional fibers that connect the nine basal body triplet microtubules to the plasma membrane. Given the accumulation of IFT complexes at the distal end of these structures, it has been suggested that the transitional fibers serve as a docking site or staging area for IFT particle formation prior to entry into the organelle [3].