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PARENT-OF-ORIGIN GROWTH EFFECTS AND THE EVOLUTION OF HYBRID INVIABILITY IN DWARF HAMSTERS

Thomas D. Brekke^{1,2} and Jeffrey M. Good^{1,3*}

¹Division of Biological Sciences, The University of Montana, Missoula Montana, 59812

Abstract

Mammalian hybrids often show abnormal growth, indicating that developmental inviability may play an important role in mammalian speciation. Yet it is unclear if this recurrent phenotype reflects a common genetic basis. Here we describe extreme parent-of-origin dependent growth in hybrids from crosses between two species of dwarf hamsters, *Phodopus campbelli* and *P. sungorus*. One cross type resulted in massive placental and embryonic overgrowth, severe developmental defects, and maternal death. Embryos from the reciprocal cross were viable and normal sized but adult hybrid males were relatively small. These effects are strikingly similar to patterns from several other mammalian hybrids. Using comparative sequence data from dwarf hamsters and several other hybridizing mammals, we argue that extreme hybrid growth can contribute to reproductive isolation during the early stages of species divergence. Next we tested if abnormal growth in hybrid hamsters was associated with disrupted genomic imprinting. We found no association between imprinting status at several candidate genes and hybrid growth, though two interacting genes involved in embryonic growth did show reduced expression in overgrown hybrids. Collectively, our study indicates that growth-related hybrid inviability may play an important role in mammalian speciation but that the genetic underpinnings of these phenotypes remain unresolved.

Keywords

reproductive isolation; genetic imprinting; Haldane's rule; speciation; *Phodopus*

Introduction

Considerable progress has been made on understanding the evolution of genetic interactions that lead to reduced fertility or viability of hybrids (i.e., intrinsic postzygotic reproductive isolation). These efforts have yielded several fundamental insights into the evolution of intrinsic reproductive isolation (reviewed in Coyne and Orr 2004), including that hybrid incompatibilities often result from deleterious interactions between divergent genes (Dobzhansky 1937; Muller 1942; Orr 1996; Brideau et al. 2006; Tang and Presgraves 2009) and that epistatic interactions involving the sex chromosomes evolve very rapidly (Coyne and Orr 1989b; Masly and Presgraves 2007). Some progress has also been made in linking genetic patterns underlying hybrid male sterility to the disruption of specific developmental

²thomas.brekke@umontana.edu, 406-243-6775. ^{3*}Corresponding author: jeffrey.good@umontana.edu, 406-243-5771.

processes during spermatogenesis (Good et al. 2010; Meiklejohn et al. 2011; Campbell et al. 2013; Bhattacharyya et al. 2013). However, much less headway has been made on the evolution of inviability and we remain relatively ignorant to the simple question of whether specific developmental pathways are predisposed to disruption in animal hybrids (Coyne and Orr 2004).

It is unclear whether we should even expect hybrid inviability to manifest during early development as embryogenesis tends to be widely conserved between species (Coyne and Orr 2004). Consistent with this, hybrid lethality tends to evolve more slowly than hybrid sterility (Coyne and Orr 1989a; 1997), leading some to question the relevance of inviability to the early stages of speciation (Sobel et al. 2010). Nonetheless, regulatory changes influencing diverse aspects of morphological development can evolve very rapidly (Abzhanov et al. 2004; Shapiro et al. 2004; Mallarino et al. 2012), and thus could also play an important role in the evolution of reproductive isolation between closely related species. In particular, the mammalian radiation is a compelling system in which to study the evolution of development in the context of speciation. Mammals show great morphological diversity between species, hybrid inviability arises at a comparatively rapid rate (Prager and Wilson 1975; Fitzpatrick 2004), and several mammalian hybrids show abnormal growth (Table 1; Gray 1972). Interestingly, many of these hybrids show parent-of-origin dependent growth where reciprocal hybrids differ in size and are either larger or smaller than the parent species (Dawson 1965; Allen 1969; Rogers and Dawson 1970; Allen et al. 1993; Zechner et al. 1996). The recurrent nature of these phenotypes suggests a common developmental and/or genetic basis, yet their relevance to mammalian speciation remains unclear because they have not been considered in an evolutionary context. Indeed, some of the best-known examples of hybrid growth effects come from hybridizing species pairs separated by millions of years of divergence (e.g., lions \times tigers; \sim 4 MY to the most recent common ancestor; Davis et al. 2010). To resolve these issues, we need to determine if the evolution of growth-related hybrid inviability in mammals reflects the disruption of a common developmental process and if the underlying intrinsic incompatibilities evolve rapidly enough to play a role in mammalian speciation.

Most of the evidence for abnormal growth in mammals derives from casual descriptions of captive hybrids that showed extreme adult sizes relative to their parent species (Gray 1972). However, a few in depth studies have shown that hybrid growth effects are associated with abnormal placentation during mid-gestation (Dawson 1965; Rogers and Dawson 1970; Zechner et al. 1996; Vrana et al. 1998). The placenta is derived largely from embryonic tissue and acts as a conduit for the transfer of maternal nutrients to the embryo. Both growth factors and their antagonists (i.e., growth repressors) are expressed in the placenta. The dosage-dependent interaction between these two classes of genes influences nutrient allocation and ultimately regulates growth in developing embryos (Haig 1996; Reik et al. 2003; Saukkonen 2004). Moreover, many placental expressed genes are controlled by an unusual mode of regulation called genomic imprinting, which results in the epigenetic silencing of one allele depending on its parent of origin (Surani et al. 1990). Imprinted genes are commonly involved in placental formation (Piedrahita 2011) and show a functional bias where paternally expressed (i.e., maternally imprinted) genes tend to promote embryonic growth while maternally expressed (i.e., paternally imprinted) genes repress growth

(Morison et al. 2005). Thus, abnormal growth in hybrid mammals may generally reflect disrupted placental function caused by failed interactions between imprinted genes (Vrana 2007).

There are three key reasons why imprinted placental genes are intriguing in the context of speciation. First, the placenta is likely subject to intense evolutionary conflict over resource allocation (Burt and Trivers 1998) because it mediates interactions between two different genomes (maternal and paternal/offspring). Conflict is particularly relevant in the case of multiple paternity (Haig 1999) where offspring strategies should evolve to garner more resources at the expense of their half-siblings and maternal countermeasures are expected to assure even allocation to all offspring (Zeh and Zeh 2000; Haig 2002; Crespi and Semeniuk 2004). Consistent with these predictions, the placenta shows the highest rate of structural evolution of all mammalian tissues (Leiser and Kaufmann 1994). Second, loss of imprinting at a single gene can skew the dosage balance between growth factors and repressors, causing abnormal development and pronounced growth (Li et al. 1999). In turn, divergence of imprinting patterns between species is predicted to cause dosage imbalance in hybrids (Varmuza 1993). Third, imprinted genes are expressed from a single chromosome and are thus functionally haploid. Similar to the well-known differential exposure of recessive X-linked hybrid incompatibilities in males (Muller 1942; Turelli and Orr 1995; 2000), haploid expression could expose recessive incompatibilities that would otherwise be masked in hybrids. Furthermore, epistatic interactions between recessive hybrid incompatibilities would also be differentially exposed in F1 hybrids because many paternally and maternally imprinted genes directly interact (Czech 1989; Haig and Graham 1991).

Patterns of placental gene expression have been evaluated in three systems that show abnormal hybrid growth. Disrupted gene expression is associated with parent-of-origin effects on morphology and size of the placenta in both deer mice (Vrana et al. 1998; Duselis and Vrana 2007; 2010) and house mice (Zechner et al. 1996; 1997; Shi et al. 2004; Brown et al. 2012). These two systems show similar placental phenotypes that appear to have different genetic bases (Zechner et al. 2004). Placental dysgenesis in deer mice is caused by an epistatic interaction between loss of imprinting in at least one paternally expressed gene and the maternally expressed X chromosome (Vrana et al. 2000; Loschiavo et al. 2007), though widespread loss of maternal imprinting is also apparent in overgrown offspring (Vrana et al. 2000). Reciprocal growth effects in the placenta of hybrid house mice also are caused by an X-autosome interaction (Zechner et al. 1996; Hemberger et al. 1999) and there is some evidence for disrupted imprinting (Shi et al. 2004; 2005). However, these regulatory effects appear to be less pronounced and genetically distinct from those described in deer mice (Zechner et al. 2004). In a third system, a hybrid cross between horses and donkeys results in parent-of-origin effects for abnormal placental morphology (Allen 1969; Allen et al. 1993), but with no evidence for disrupted genomic imprinting (Wang et al. 2013). As these three systems show similar phenotypes caused by different genetic mechanisms, it remains to be seen how often disrupted imprinting underlies the evolution of mammalian reproductive isolation in general and parent-of-origin growth effects in particular.

Here we describe patterns of reproductive isolation between two species of dwarf hamsters, *Phodopus sungorus* and *P. campbelli*. Dwarf hamsters are native to the xeric habitats of central Asia with *P. sungorus* inhabiting the Kazakh steppe (Ross 1998) and *P. campbelli* inhabiting the semi-deserts of Mongolia, northern China, and southern Russia (Ross 1995). They are sister species (Neumann et al. 2006) that have only recently been elevated from subspecies status based primarily on evidence of sterility in hybrid males (Sokolov and Vasil'eva 1993). The two species have very similar karyotype morphologies ($2n=28$), though hybrid male sterility is associated with extensive autosomal and sex chromosome asynapsis during meiosis I (Safronova and Vasil'eva 1996; Safronova et al. 1999). In addition, one direction of the cross (female *P. sungorus* \times male *P. campbelli*) has been reported to result in "heterotic" hybrids with exaggerated growth and an increased incidence of birth defects (Safronova and Vasil'eva 1996). These observations suggest that dwarf hamsters may provide a novel system with which to evaluate the developmental basis of abnormal hybrid growth between mammal species still in the early stages of divergence. We have three primary objectives. First, we use reciprocal crosses to test for hybrid inviability phenotypes in dwarf hamsters, focusing on parent-of-origin growth effects throughout the lifecycle of F_1 hybrids. Second, we examine levels and allele-specific patterns of expression at eight candidate genes to test for disrupted expression and/or genomic imprinting in hybrid placenta. Third, we present DNA divergence estimates between dwarf hamsters and other species pairs exhibiting disrupted hybrid growth to assess the evolutionary tempo of growth-related hybrid inviability in mammals.

Methods

Animals

Outbred dwarf hamster colonies were established at the University of Montana in the fall of 2011 using six mating pairs of *P. campbelli* provided by Robert Johnston and six mating pairs of *Phodopus sungorus* provided by Ned Place, both from Cornell University. These stocks were derived from natural populations sampled by Catherine Wynne-Edwards in 1981 and most recently supplemented with additional wild hamsters in 1990 (Scribner and Wynne-Edwards 1994). We have maintained our breeding colonies using a crossing scheme designed to minimize inbreeding (Wright 1921). All animals were housed in 14L:10D light/dark regimen and in accordance with IACUC regulations.

Experimental crosses and phenotypic analyses

We conducted a total of 331 experimental crosses within and between the two species: 1) 110 *P. campbelli* \times *P. campbelli*, 2) 88 *P. campbelli* \times *P. sungorus*, 3) 32 *P. sungorus* \times *P. campbelli*, and 4) 101 *P. sungorus* \times *P. sungorus*. Here and elsewhere the female is given first following standard mouse genetics crossing notation. These crosses were used to collect a suite of developmental phenotypes described below.

First, we collected late-term embryos and placentas from euthanized pregnant females to determine the frequency and extent of developmental defects. Dwarf hamsters have discoid, labyrinthine hemochorial placentas (Elliot and Crespi 2009) with an 18-day gestation period and a facultative delay of up to four days due to developmental diapause and/or delayed

implantation (Newkirk et al. 1997). To control for this variation, dissected embryos were developmentally staged according to a suite of established characters in golden hamsters (Boyer 1953) and mice (Butler and Juurlink 1987). For our analysis, we only used late-term embryos corresponding roughly to Theiler's Stages 24-27 of mouse development (Theiler 1972). Litter size was recorded and dissected embryos and placentas were photographed, weighed and given a presence/absence score for several developmental defects including the occurrence of molar conceptuses (hydatiform moles), embryo reabsorption, and embryo swelling (edema). All embryos and placenta were then snap-frozen on dry ice to preserve RNA for gene expression analyses.

Second, we allowed several crosses to proceed to term to determine if growth phenotypes identified *in utero* persisted throughout the animal's life cycle and to test for the emergence of new phenotypes in adults. To quantify mating isolation, we tested for differences in the number of successful crosses and latency to birth for adult females paired with a hetero- or conspecific male for up to 40 days. To quantify postnatal growth, we generated a standard growth curve for each cross type by weighing each offspring every ten days after birth until day 100. We modeled growth with an asymptotic curve and tested for differences in the asymptote (final adult size) between each of the cross types. Female *P. sungorus* × male *P. campbelli* hybrids could not be brought to term and were excluded from these experiments.

Phenotypic data has been deposited in Dryad and all statistical analyses were performed using R version 3.0.2 (R Core Team 2008). We calculated both one-way analyses of variance (ANOVA) and non-parametric pairwise Wilcoxon rank-sum tests for all comparisons between cross types. Results of the Wilcoxon test are reported for phenotypes with large differences in variance between the groups (e.g., embryo and placental weights). Multiple comparisons were accounted for by using a Bonferroni correction when appropriate.

Genetic sex-typing of embryos

The sex of hybrid embryos was determined by Polymerase Chain Reaction (PCR) amplification and Sanger sequencing of a 764 bp fragment of the X-linked gene *Zfx*. Degenerate primers were designed for dwarf hamsters by modifying the generic LGL331 and LGL335 primers of Shaw and colleagues (2003) based on an alignment of *Zfx* and *Zfy* sequences from rat, house mouse, guinea pig, Golden hamster, and Chinese hamster (see Supplemental Table 2 for accession numbers). The *Zfx/Zfy* sex-typing system usually relies upon a diagnostic intron length polymorphism between homologous genes on the X (*Zfx*) and Y (*Zfy*) (Shaw et al. 2003), but we were unable to amplify *Zfy* in *Phodopus*. Therefore, we sequenced *Zfx* and identified five fixed nucleotide differences between the species that we then assayed in hybrids. Heterozygous hybrids were classified as female and homozygous hybrids possessing the expected maternal genotype were classified as male. We verified the accuracy of our assay by typing several adult hybrids of known sex; however, the sex of non-hybrid individuals could not be determined using this approach. All primer sequences and PCR reaction conditions used in this study can be found in Supplemental Table 2. Sequence alignments were performed using the program Geneious (Drummond et al. 2005).

Survey of candidate gene expression

We targeted eight genes that show imprinted expression in the placenta of house mice (Morison et al. 2005), including four paternally expressed genes (maternally imprinted *Igf2*, *Mest*, *Peg3*, *Snrpn*) and four maternally expressed genes (paternally imprinted *H19*, *Igf2r*, *Grb10*, and *Mash2*). These candidates were selected because several of them show disrupted placental imprinting in hybrid deer mice (Vrana et al. 1998). Primers were designed using Primer3 (Rozen and Skaletsky 2000) based on exon sequences aligned between mouse, human, rat, and guinea pig (Supplemental Table 2). Amplicons were designed to span at least one intron in five of the genes to minimize the risk of genomic DNA contamination. PCR products for *Peg3*, *H19* and *Mash2* did not span introns because either no conserved priming sites could be found or no diagnostic site was present near the intron-exon boundary.

We assayed allele-specific expression of these genes by sequencing complementary DNA (cDNA) from 18 late-gestation placentas, including three from each species and six (three male, three female) from each reciprocal hybrid. Whole placentas were homogenized in liquid nitrogen with a mortar and pestle and total RNA was extracted using an E.Z.N.A. Total RNA Kit (Omega), treated with DNase, and converted to cDNA with the cDNA Supermix Kit (Quantas). Exonic regions were then PCR amplified from cDNA, Sanger sequenced, and examined for fixed differences between the species. All eight loci are autosomal in house mice; therefore hybrid individuals should be heterozygous at all diagnostic positions in the absence of imprinting. Using this rationale, we classified gene expression in hybrids as imprinted (homozygous for the maternal or paternal allele) or biallelic (heterozygous). As with the sex-typing assay, this assay is only effective in the F₁ hybrids. Imprinted expression was called only when a single peak from the expected allele was visible on the chromatogram (Supplemental Figure 1). This is a conservative metric given that imprinting sometimes results in skewed biallelic expression (Babak et al. 2008). To evaluate the sensitivity of our assay to detect skewed expression, we tested each primer pair by sequencing a series of “mock hybrid” cDNA pools constructed by combining *P. campbelli* and *P. sungorus* cDNA in the following ratios: 1:99, 25:75, 50:50, 75:25, and 99:1. All PCR products were Sanger sequenced at the University of Montana Murdock Lab DNA Sequencing Facility or the University of Arizona Genetics Core and have been deposited in GenBank under the accession numbers JX217832-JX217849, JX436485-JX436486, and KF673394.1-KF673395.1.

We assayed relative expression level using a DyNAmo Flash SYBR Green qPCR kit (Thermo Scientific) on an Agilent Mx3000 in six of each hybrid type and six of each parent species. ROX was used at a concentration of 0.3X as a passive reference dye. All primer pairs had efficiencies between 90% and 105%, which were determined with a four point dilution series. All reactions were carried out in triplicate in 20 μ l reaction volumes with final primer concentrations of 1mM. Quantitative PCR reaction conditions are reported in Supplemental Table 2. Melt curves were collected after 40 cycles and single peaks were found for all genes. The gene *Ywhaz* was used as a normalizing control (Kumar et al. 2012). Crossing thresholds (C_T) were generated with Agilent's MxPro software and analyzed with custom R scripts using the C_T method (Schmittgen and Livak 2008).

Genetic divergence between hybridizing mammal species

We compiled mitochondrial cytochrome *b* (cyt *b*) sequence data for 36 species pairs that have been reported to show abnormal hybrid growth (Supplemental Table 1). For dwarf hamsters, we designed primers to amplify and sequence 910 base pairs (bp) of cyt *b* in both species (Supplemental Table 2). For the other 35 species pairs we used previously published cyt *b* sequences from GenBank (see Supplemental Table 1 for accession numbers); five species pairs did not have available data for cyt *b*. After trimming positions with missing data, 34 species pairs shared a common 718 bp alignment that we used to calculate Kimura two-parameter-corrected pairwise divergences (Dxy) with the program Phylip (version 3.6a3, Felsenstein 2002).

We also calculated pairwise synonymous divergence (K_s) between *P. campbelli* and *P. sungorus* using partial coding sequences from the eight nuclear genes sequenced in our expression assays. For comparison, orthologous sequences were retrieved from available genomic data from *Mus musculus* and *M. spretus* (Keane et al. 2011). Each nuclear sequence was aligned, trimmed to the same length and codons were assigned. Then the genes were concatenated into a 5,669-base fragment to calculate K_s with the program DNAsp (version 5.10.1, Librado and Rozas 2009).

Results

Reduced pregnancy rate in hybrid crosses

Pregnancy rates were similar and relatively high within each species. Females became pregnant in 83% (67 of 81) of *P. campbelli* crosses and 84% (68 of 81) of *P. sungorus* crosses. In contrast, *P. campbelli* females were successfully impregnated by a *P. sungorus* male only 68% of the time (43 of 63 crosses; $P=0.017$, Fisher's Exact Test (FET) versus pooled species, Bonferroni corrected $\alpha=0.025$). The pregnancy rate was also marginally reduced in the reciprocal cross (*P. sungorus* \times *P. campbelli*; 66% or 19 of 29 crosses; $P=0.038$ FET versus pooled species). When considering the subset of pairs that reached parturition, we found no reduction in the average latency from pairing to birth for successful heterospecific pregnancies relative to conspecific matings. *Phodopus campbelli* and *P. sungorus* averaged 24.2 days (62 crosses) and 23.3 (63 crosses) days respectively from pairing to birth, while the hybrid cross *P. campbelli* \times *P. sungorus* averaged 22.2 days (37 crosses). The reciprocal hybrid cross, *P. sungorus* \times *P. campbelli*, did not yield any successful births.

Parent-of-origin effects with extreme asymmetric hybrid overgrowth

We found that the mean weight of hybrid embryos from a *P. sungorus* mother and a *P. campbelli* father was ~38% larger than any other cross type (Figure 1A, $P<0.001$, Wilcoxon rank-sum test, Bonferroni corrected $\alpha=0.008$). Parent-of-origin dependent growth was even more striking in the placenta (Figure 1B, Supplemental Figure 2); placentas derived from a female *P. sungorus* and a male *P. campbelli* father were ~300% heavier than placentas from any other cross type (Figure 1B, $P<0.001$, Wilcoxon rank-sum test, Bonferroni corrected $\alpha=0.008$). We found no sex-specific differences in the placenta or embryo weights of either reciprocal hybrid type (*P. sungorus* \times *P. campbelli*: placenta $P=0.175$, embryo $P=0.109$; *P.*

campbelli × *P. sungorus*: placenta $P=0.880$, embryo $P=0.880$; Wilcoxon rank-sum tests, Bonferroni corrected $\alpha=0.013$). Following Vrana and colleagues (1998) we will hereafter refer to the large *P. sungorus* × *P. campbelli* hybrids as “S×C” and the reciprocal *P. campbelli* × *P. sungorus* hybrids as “cxs”, where the species are designated by the first letter of their specific epithet, the maternal species is listed first, and the capitalization reflects the relative size of the hybrid offspring.

The overgrown S×C offspring were more often affected by severe developmental defects. We found an elevated proportion of molar conceptuses and reabsorbing embryos in S×C crosses relative to all other cross types (Table 2). Also known as hydatiform moles, molar conceptuses are a form of placental pathology characterized by excessive extra-embryonic (placenta) tissue and no embryonic tissue (Supplemental Figure 2D; Lindor et al. 1992). Twenty-five percent of S×C embryos (18 of 73) showed edema, characterized by mild to extreme swelling (Supplemental Figure 2C), whereas embryonic edema was comparably rare in all other crosses (Table 2). Thus, abnormal *in utero* development was largely restricted to S×C hybrids, with ~70% (53 out of 73) of S×C embryos afflicted by severe developmental defects (even when excluding embryos with edema, S×C offspring were significantly overgrown). In contrast, cxs hybrids were not significantly different than *P. campbelli* or *P. sungorus* for any of the *in utero* developmental phenotypes that we considered. However, late-gestation hybrid litters were smaller than intraspecific litters (Table 2).

The lack of significant undergrowth phenotypes in late-term cxs hybrids (Figure 1) contrasts with described parent-of-origin effects in hybrid deer mice (Vrana et al. 1998) and house mice (Zechner et al. 1996), where significant but opposite growth effects are manifest *in utero* in reciprocal hybrid embryos and/or placentas. Our study lacks power to detect subtle weight differences at late gestation (e.g., we have only ~12% power to detect an effect size of 0.2). Because parent-of-origin effects often persist or even become exaggerated into adulthood, we next compared the postnatal growth curves of both species to the cxs hybrids (Figure 2). This experiment was initiated with 26 individuals from 6 crosses for *P. campbelli*, 38 individuals from 6 crosses for *P. sungorus*, and 45 individuals from 15 crosses for *P. campbelli* × *P. sungorus* hybrids. Though approximately the same size at late gestation (Figure 1A), adult cxs hybrids were much smaller than either parent species (Figure 2; $F_{2,14}=77.116$, $P<0.001$). Specifically, we found a significant reduction in adult weights of cxs males versus males of either species (Figure 3; $P<0.001$, *t*-test, Bonferroni corrected $\alpha=0.017$). As is typical for mammals, dwarf hamsters are sexually dimorphic as adults (80 days) with males ~10% larger than the females (Figure 3). In contrast, cxs hybrid females were larger than hybrid males (Figure 3); 80-day old cxs females were approximately the same size as their *P. campbelli* mothers whereas the males were only half as large as their fathers (Figure 3). Thus, reduced adult growth of cxs hybrids (Figure 2) appears to be driven mostly by a male-specific reduction in body weight. We did not measure postnatal growth of S×C hybrids because three attempts to birth the overgrown hybrids failed and resulted in maternal death.

Reduced postnatal growth only in cxs hybrid males is consistent with Haldane's rule, which states that inviability should affect males more often than females in male-heterogametic

taxa (Haldane 1922). To further test for sex-specific inviability we analyzed the sex ratios of late-term and adult hybrids. We found no significant bias in the sex ratio of either hybrid type *in utero* (Table 2; cxs $\chi^2=1.125$, $df=1$, $P=0.288$; S×C $\chi^2=0.118$, $df=1$, $P=0.537$, chi-squared test), but we did find significantly male-biased adult sex ratios in cxs hybrids (Table 3; 61.8% male, $\chi^2=8.463$, $df=1$, $P<0.001$, chi-squared test). Further inspection of the average counts for each sex suggests that this male-biased skew primarily reflects a reduction in the number of females per litter (Table 3). Finally, we tested whether one sex was more susceptible to molar conceptuses, reabsorption, and/or edema in the S×C hybrids. We found approximately equal numbers of males and females affected by each of these phenotypes (molar conceptuses: $P=0.715$; reabsorbing embryos: $P=1.0$; edema: $P=0.169$, $n=25$ females and 30 males, FET). Thus, we find a surprising pattern in adult cxs hybrids where males are more common but significantly smaller than females.

No widespread disruption of imprinting associated with parent-of-origin growth effects

The existence of strongly asymmetric placental growth phenotypes between reciprocal hybrids (Figure 1) provides a powerful framework with which to differentiate between gene expression changes associated with abnormal growth versus differences that manifest between all hybrid genotypes and the parental species. Species-specific disruption of placental imprinting has been put forth as a general explanation for such parent-of-origin dependent growth effects in reciprocal hybrids (Vrana et al. 1998; Vrana 2007). Specifically, this dosage model predicts that (i) hybrid overgrowth results from maternal expression of one or more growth factors that are normally silenced through imprinting and (ii) that undergrowth results in the reciprocal cross when growth repressors are expressed from the normally silenced paternal genes. For a given gene, this simple model predicts that disrupted imprinting will result in biallelic expression in one hybrid while expression in the reciprocal hybrid remains properly imprinted. Extreme overgrowth occurs in our dwarf hamster crosses when *P. sungorus* is the mother and undergrowth is manifest when *P. sungorus* is the father, albeit undergrowth is not apparent until after birth (Figures 1 and 2). Thus, if disrupted imprinting is the cause of these parent-of-origin effects then we should see the gain of expression of *P. sungorus* (maternal) growth factor alleles in S×C placentas, *P. sungorus* (paternal) growth repressors in cxs placentas, and proper imprinting of *P. campbelli* alleles in both hybrid cross types. Gene expression patterns that are the same between the reciprocal hybrids are by definition not associated with parent-of-origin dependent phenotypes.

To test this general model, we assayed the expression of eight genes that are imprinted in mice and have been shown to influence embryonic growth. For each gene, we tested six placentas (three per sex) from each of the reciprocal hybrids for imprinted (monoallelic) expression of the maternal or paternal allele (Supplemental Figure 1). Seven of the eight candidate genes were found to contain one or more fixed differences between the species. The paternally expressed gene *Mest* showed no fixed differences and therefore could not be assayed for allele-specific expression in hybrids. The maternally imprinted candidate *Igf2* showed expression of only the paternal allele, consistent with imprinted expression patterns in *Mus* (Table 4). Two maternally imprinted genes, *Peg3* and *Snrpn*, showed proper imprinting in cxs hybrids, but exhibited some variation in imprinting status among S×C hybrids. In *Peg3*, one male and one female S×C hybrid showed biallelic expression while

the other four offspring only expressed the paternal allele. In *Snrpn*, a single Sx C hybrid female showed biallelic expression while the other five individuals showed proper imprinting. Three of the four maternally expressed genes (*Grb10*, *Igf2r*, and *Mash2*) showed biallelic expression in both reciprocal hybrids, while *H19* showed only maternal expression (Table 4, Supplemental Figure 1; Morison et al. 2005).

Patterns of allelic expression were identical between males and females for all genes except at *Snrpn* as noted above. Sequencing results from our constructed mock hybrid pools confirmed that the species-specific alleles amplified with similar efficiency for all genes. We found approximately equal allele representation at diagnostic positions in 50:50 mock hybrid pools. Skewed biallelic expression was detected for reciprocal 75:25 pools while 99:1 pools were scored as monoallelic, indicating that our assays were sensitive to detecting biallelic expression at genes with moderately (but not highly) skewed allele-specific expression.

We used qPCR to test for changes in expression level in the same eight candidate genes. These assays test for changes in expression level irrespective of changes in imprinting status and so provide an additional means by which to test for regulatory disruption in the hybrids. Three genes (*Grb10*, *H19*, *Mest*) showed similar expression between the species and the reciprocal hybrids (Figure 4; *Grb10*, $F_{3,20}=2.158$, $P=0.125$; *H19*, $F_{3,20}=0.754$, $P=0.533$; *Mest*, $F_{3,20}=1.733$, $P=0.193$). *Snrpn* showed significantly different expression between the species with the hybrids showing intermediate expression ($F_{3,20}=3.205$, $P=0.045$). In all cases, cxs hybrids showed similar expression to at least one of the parental species. *Peg3* tended towards higher expression in both hybrids, but this difference was only significant relative to *P. sungorus* ($F_{3,20}=4.750$, $P=0.012$). *Igf2* and *Igf2r* were the only genes that showed significantly different expression between the reciprocal hybrids, and in both cases the cxs hybrid was similar to the parent species (*Igf2*, $F_{3,20}=5.749$, $P=0.005$; $F_{3,20}=3.913$, $P=0.024$ for *Igf2r*).

Divergence estimates for taxa showing abnormal growth effects

We estimated Dxy (Kimura-two-parameter-corrected) sequence divergence for 34 species pairs that show abnormal hybrid growth and had *cyt b* sequence data available from GenBank. Pairwise divergence estimates ranged between 19.3% and 0.0% (Supplemental Table 1). Mitochondrial divergence for dwarf hamsters at *cyt b* was 3.7%. We also estimated pairwise silent site divergence (K_s) for dwarf hamsters and house mice (*M. musculus* and *M. spretus*) in a concatenated alignment of eight candidate nuclear genes to be 0.9% and 1.4%, respectively.

Discussion

Parent-of-origin growth effects in dwarf hamsters and other mammals

Dwarf hamster hybrids display strong parent-of-origin growth effects that manifest a wide range of inviability phenotypes. When a *P. sungorus* female was crossed with a *P. campbelli* male, embryo and placenta overgrowth was so extreme that it was ultimately lethal to the mother and offspring during birth. However, despite the potentially high rates of maternal mortality suggested by our study, viable SxC hybrids have been reported (Sokolov and

Vasil'eva 1993). In this previous study, the average adult weight of 13 S×C hybrids was 55.2 grams — nearly 200% the weight of either species. It is unclear what the probability of a successful S×C birth is based on these limited data, but they do demonstrate that the striking placental and embryonic overgrowth that we observed during late gestation (Figure 1) persist through to adulthood. Alternatively, adult cxs hybrid males were approximately 40% smaller than their male parents though they appeared normal at birth with respect embryo and placenta sizes. Thus the overgrown S×C cross yields more severe hybrid inviability phenotypes but both cross types show evidence for growth effects and reproductive isolation as indicated by significantly reduced litter sizes (Table 2).

Parent-of-origin growth effects in hybrid dwarf hamsters are strikingly similar to several previously described examples in other hybrid mammals (Vrana 2007). Extreme and often lethal hybrid overgrowth also occurs in crosses between female *Peromyscus polionotus* and male *P. maniculatus* (Dawson 1965; Rogers and Dawson 1970; Dawson et al. 1993; Vrana 2007). Likewise, the well-known example of reciprocal crosses between lions (*Panthera leo*) and tigers (*P. tigris*) results in strong parent-of-origin growth phenotypes that persist into adulthood. So-called ligers (hybrids from a female tiger × male lion) reach adult weights approaching 150% the size of a tiger (the larger of the parents). Gray (1972) quotes a report of one liger that “weighed as much as both parents together”, which is striking if not strongly quantitative. The reciprocal cross (i.e., tigons) is smaller than either species (Vrana 2007). No data are available for liger or tigon placentas but we predict that their growth would be similarly affected.

Interestingly, not all hybrid crosses presenting abnormal placental growth also manifest embryonic or adult growth phenotypes. For example, parent-of-origin developmental effects in several mouse crosses (genus *Mus*) are restricted to the placenta, and do not strongly influence embryonic or adult growth (Zechner et al. 1996; Kurz et al. 1999; Zechner et al. 2004). Reciprocal crosses between horses and donkeys also yield parent-of-origin effects on placental size and morphology but not embryo size (Allen 1969; Allen et al. 1993). In both of these systems abnormal placentation impacts embryonic viability in hybrids (West et al. 1977; Zechner et al. 1996; Kurz et al. 1999; Allen 2001). Artificial insemination has recently been used to achieve a more divergent *Mus* cross that results in extreme placental and embryonic growth (*M. musculus* and *M. caroli*; Brown et al. 2012). Thus abnormal placentation appears to represent an important but not sufficient first step in the evolution of parent-of-origin growth effects in adult hybrid mammals. Most of the phenotypic data from mammalian hybrids derive from qualitative differences in postnatal body size (Gray 1972) and placental phenotypes are rarely collected. Therefore, it is possible that the disruption of hybrid placentation is much more rapidly evolving and widespread than is commonly appreciated. Consistent with this prediction, the rate at which reproductive isolation evolves across different mammal groups has been shown to correlate with physiological aspects of placental morphology (Elliot and Crespi 2006).

The hybrid growth effects noted by Gray (1972) derive from crosses between species pairs spanning a broad range of taxonomic (intraspecific to intergeneric) and genetic divergence (0 to ~20% pairwise divergence at *cyt b*; Supplemental Table 1). Some of these qualitative growth effects are anecdotal and require further validation. Other examples likely reflect

heterosis generated through the masking of deleterious recessive alleles and thus do not reflect true intrinsic incompatibilities. In this context, parent-of-origin growth effects likely provide the strongest and most relevant examples of hybrid inviability. The *P. sungorus* × *P. campbelli* cross is especially intriguing because they are among the most closely related species that show asymmetric hybrid growth (3.7% pairwise divergence at *cyt b*). This is on the low end of divergence typically found between sister mammalian species (Bradley and Baker 2001). By comparison, deer mice (*P. polionotus* and *P. maniculatus*; 4.0%), horses and donkeys (7.7%), lions and tigers (11.6%), and house mice (*M. musculus* and *M. spretus*; 9.6%) are all more divergent. Admittedly, mitochondrial DNA often does not accurately reflect genomic divergence between species (Ballard and Whitlock 2004) but information on nuclear divergence is not yet available for most of the species pairs considered here. Pairwise synonymous divergence (K_s) between *P. sungorus* × *P. campbelli* at the eight candidate imprinted genes was 0.9%, indicating that they also show very low divergence across the nuclear genome. For comparison, the same eight genes show considerably more synonymous divergence ($K_s=1.4\%$) between two house mouse species (*M. musculus* and *M. spretus*) estimated to share a most recent common ancestor ~1.5 MYA (Suzuki et al. 2004). These data indicate that parent-of-origin growth effects can manifest between closely related species and may contribute to the early stages of mammalian speciation.

Our data also support the observation that the evolution of abnormal hybrid growth in mammals tends to follow Haldane's rule. Hybrid mice, hamsters, and deer mice all show male-specific growth phenotypes at some point during development. Deer mouse hybrids show a strongly female-biased sex ratio (Dawson et al. 1993) due to more extreme overgrowth in male placentas and embryos (Vrana et al. 2000; Vrana 2007). Male placentas also tend to be much larger in mouse hybrids between *M. musculus* and *M. spretus* or *M. macedonicus*, (Zechner et al. 1996). Hamster hybrids do not show any sex-specific placental or embryonic differences, but adult cxs hybrid males are severely growth restricted when compared to the females (Figure 3). However, we did observe a male bias in cxs litters (~60%) that could reflect differential female inviability and thus an exception to Haldane's rule. However, we believe that a maternal effect in this cross provides the simplest explanation for this pattern. Maternal effects that give rise to skewed sex ratios are common in mammals (Clutton-Brock and Iason 1986) and there were no embryonic phenotypes that indicated females were less viable *in utero*.

Resolving the genetic and epigenetic bases of parent-of-origin growth

If we assume that abnormal hybrid growth in mammals generally follows the Dobzhansky-Muller model for intrinsic incompatibilities (Dobzhansky 1937; Muller 1942), then it is likely caused by the evolution of incompatible interactions between growth-related genes that have diverged between the hybridizing species. Such failed interactions could disrupt the epigenetic regulation of imprinting and change the expression of genes that control offspring growth. Alternatively, hybrid incompatibilities may cause abnormal growth independent of disrupted imprinting. Differentiating between these models remains a fundamental problem in mammalian speciation. Epigenetic disruption of imprinting has emerged as the predominant model to explain parent-of-origin dependent growth in mammal hybrids (Vrana 2007; Crespi and Nosil 2013). This model is compelling because errors in

imprinting have the ability to explain growth effects (many imprinted genes regulate growth), parent-of-origin effects (imprinting is a parent-of-origin dependent process), and why these phenotypes are common in mammals (mammals are the only vertebrates where imprinting has been found). Indeed, an analogous regulatory process has been described in the endosperm of angiosperms (Lin 1982; Haig and Westoby 1989). Hybrid endosperm development sometimes shows parent-of-origin growth effects (Ishikawa et al. 2011), and disrupted imprinting has been associated with abnormal endosperm development (Erilova et al. 2009). Nonetheless, despite the broad appeal of this model, no consensus has been reached on the role of disrupted imprinting in the evolution of parent-of-origin dependent growth effects in mammals (Zechner et al. 2004; Brown et al. 2012; Wang et al. 2013).

Our candidate gene data do not support the hypothesis that changes in imprinting status contribute to abnormal hybrid growth in hamsters. Imprinting is maintained at some genes in reciprocal dwarf hamster hybrids while other genes are biallelically expressed in both hybrids (Table 4). Gene expression patterns that are the same between the reciprocal hybrids cannot by definition explain phenotypic differences between those hybrids, even if they are a pleiotropic consequence of underlying hybrid incompatibilities. None of the candidate genes showed differences in imprinting status between the reciprocal hybrids. This strongly contrasts with the extensive changes in imprinting status associated with similarly extreme parent-of-origin growth effects between reciprocal deer mouse hybrids (Table 4; Vrana et al. 1998). In our study, biallelic expression of *Igf2r*, *Grb10*, and *Mash2* may simply reflect that these genes are not imprinted in hamsters or that imprinting is disrupted at these genes in hybrids independent of the observed parent-of-origin growth effects. Our qPCR results support the former hypothesis; biallelic expression of *Igf2r*, *Grb10*, and *Mash2* was not accompanied by concomitant increases in expression level in both hybrids relative to the parental species as would be predicted for loss of imprinting (Figure 4). Likewise, apparent variation in the imprinting at *Peg3* (two out of six S×C offspring have biallelic expression) and *Snrpn* (biallelic in a single S×C female) could reflect polymorphism for imprinting at these genes in dwarf hamsters. In agreement with these patterns, *Mash2* does not appear to be imprinted in deer mice (Vrana et al. 1998), imprinting of *Igf2r* is polymorphic in humans (Xu et al. 1993), and *Grb10* is imprinted in opposite parental directions in a tissue-specific manner in mice (Garfield et al. 2011). Collectively, these data argue that imprinting status is labile within and between species and underscore that much more work is needed to understand the evolution of genomic imprinting.

The limitations of our allele-specific expression assay aside, monoallelic expression at *Igf2*, *H19*, *Peg3*, and *Snrpn* clearly demonstrates that imprinting is not widely disrupted in placenta of Phodopus hybrids. Moreover, several genes with disrupted imprinting in deer mouse hybrids show normal imprinted expression in hamster hybrids (Table 4; Vrana et al. 1998). When considered in light of other studies, this result indicates that both the identity and genomic extent of disrupted imprinting is highly variable across mammalian hybrids (Vrana et al. 1998; O'Neill et al. 1998; Roemer et al. 1999; Schutt et al. 2003; Wang et al. 2013). However, our candidate gene approach does not exclude disrupted imprinting as the ultimate cause of parent-of-origin dependent growth in hamsters. Our experiment has only considered ~10% of the approximately 80 imprinted genes expressed in the mouse placenta

(Morison et al. 2005). Imprinted genes also tend to occur in clusters in mammalian genomes (Verona et al. 2003) and cluster-specific imprinting breakdown has been described in deer mice (Wiley et al. 2008). The diagnostic seven genes that we surveyed represent only five of the eighteen clusters in house mice (Morison et al. 2005). We are currently collecting genome-wide expression data to determine if cluster-specific breakdown of imprinting also occurs in hybrid dwarf hamsters.

While allele-specific expression differences in hamster hybrids did not correlate with parent-of-origin growth effects, we did observe significant differences in expression levels at *Igf2* and *Igf2r* between reciprocal hybrids (Figure 4). These genes are antagonists to each other in the fetal growth pathway. *Igf2* encodes a protein hormone (IGF2) that promotes growth while *Igf2r* encodes a transmembrane receptor that sequesters IGF2 out of circulation. The observation that two interacting genes involved in regulating fetal growth are differentially expressed in hybrids with severe growth defects is intriguing. We naively predicted that *Igf2* (and other paternally expressed growth factors) would show increased expression in overgrown hybrids both *Igf2* and *Igf2r* show lower expression in S×C. This result suggests that our simple models based on imprinted gene dosage and functions are unlikely to effectively explain the complex phenotypes of parent-of-origin growth. Recent work in house mice has shown that complex epistatic interactions between imprinted and non-imprinted genes can lead to pervasive disruption of gene regulatory networks and manifest in strong parent-of-origin effects (Mott et al. 2014). Such effects are likely to be even more pervasive when considering the extensive epistatic interactions that are exposed when two divergent genomes are combined in a hybrid.

Finally, our data also establish that sex-specific effects are recurrent in the evolution of parent-of-origin dependent hybrid growth. At face value this is not surprising given Haldane's rule (Haldane 1922) and the general predictions of dominance theory (Turelli and Orr 2000). However, sex-specific effects are not expected in the placenta because the paternal X chromosome is silenced in extra-embryonic tissues in rodents (imprinted X chromosome inactivation or XCI; Lyon 1961; 1962). Given imprinted XCI, X-linked genes expressed in the placenta are expected to be effectively hemizygous in both sexes and thus a recessive incompatibility on X chromosome should affect both sexes similarly. Several hypotheses have been proposed to account for unexpected sex-specific effects in hybrid placenta (Hemberger et al. 2001). Recessive X-linked incompatibilities may be partially masked in females due to incomplete silencing of the paternal X chromosome (i.e., leaky imprinted XCI) or through some contribution of X-linked gene products expressed in female embryos where XCI is random (Payer and Lee 2008). Likewise, disruption of imprinted XCI in the placenta could also mask deleterious recessive interactions in females; though it seems unlikely that breakdown of a major epigenetic process would generally result in increased viability. Finally, the male-specific effects could reflect the action of the Y chromosome (Hemberger et al. 2001), though Y-linked effects usually are restricted to male reproductive phenotypes. Differentiating among these potential models will be crucial for resolving the ultimate causes of male-biased developmental abnormalities in mammals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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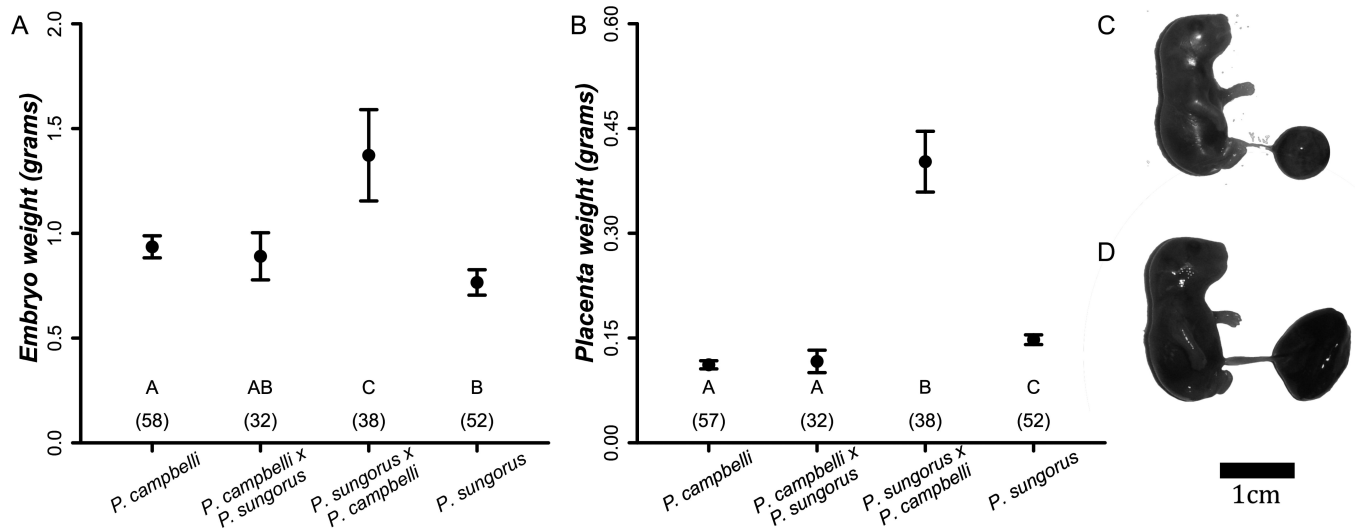


Figure 1.

Average weights in grams (± 2 SE) of late-term embryos (A) and placentas (B) were much higher in female *P. sungorus* \times male *P. campbelli* (S \times C) hybrids when compared to other cross types. Letters designate significant differences between groups based on pairwise Wilcoxon rank-sum tests and group sample sizes are in parentheses. Numbers in parentheses are sample sizes. Insets show (C) a normal *P. campbelli* offspring with average sized placenta and (D) an overgrown *P. sungorus* \times *P. campbelli* (S \times C) offspring with an enlarged placenta.

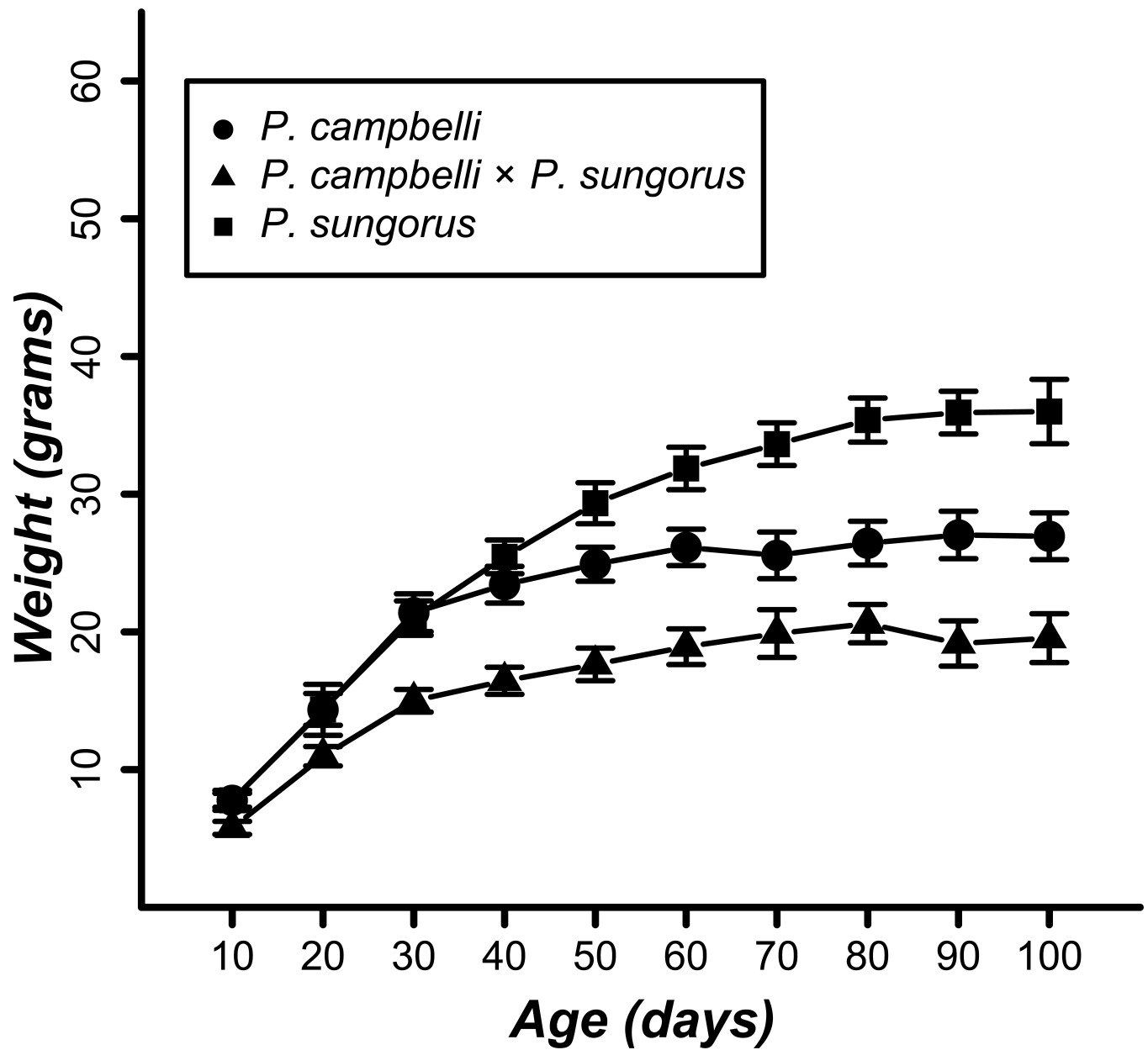


Figure 2. Growth curves for *P. campbelli*, *P. sungorus*, and female *P. campbelli* × male *P. sungorus* (cxs) offspring. Average weights (± 2 SE) are shown every 10 days. Hybrids show a significant reduction in adult size compared to the parents.

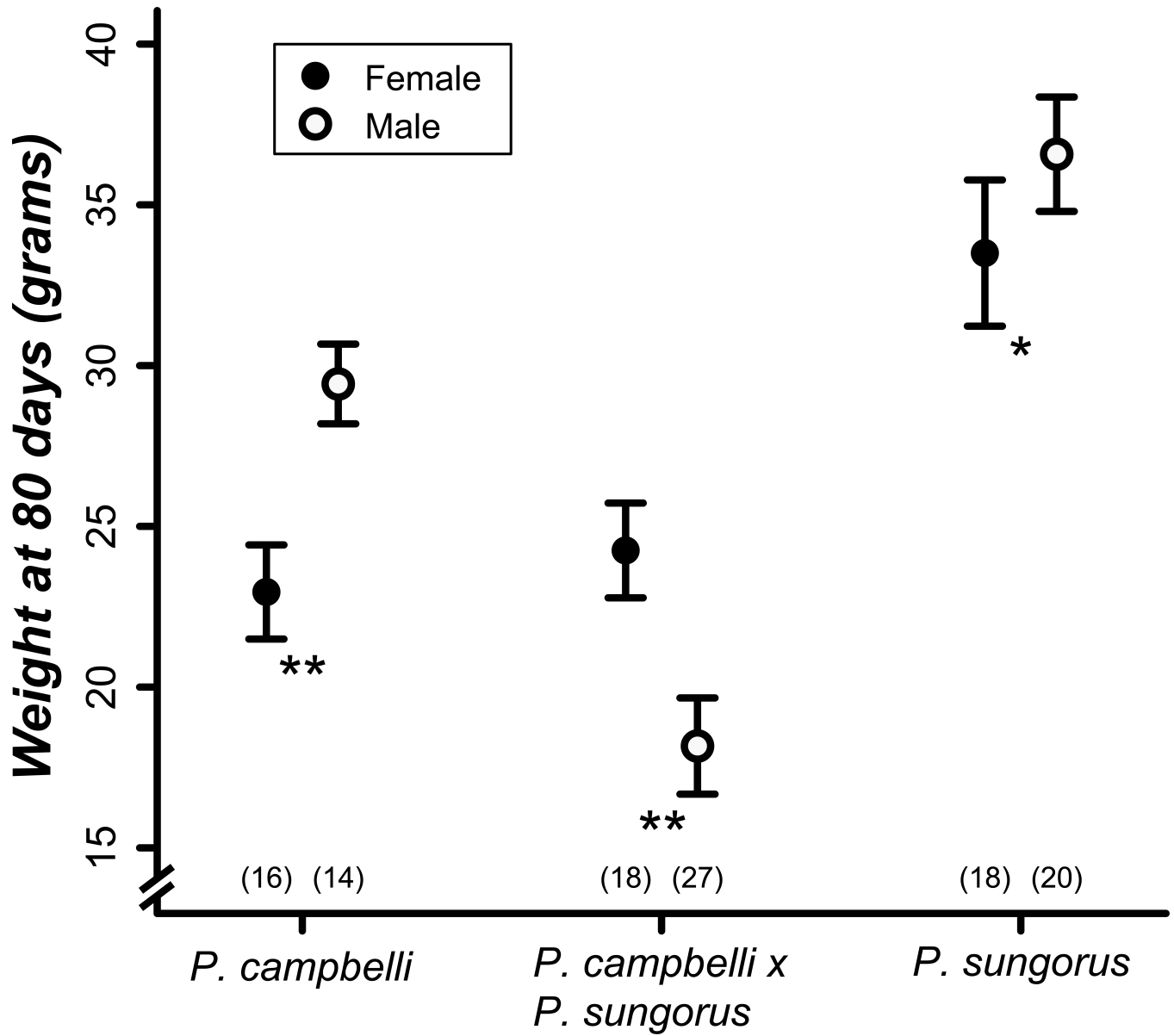
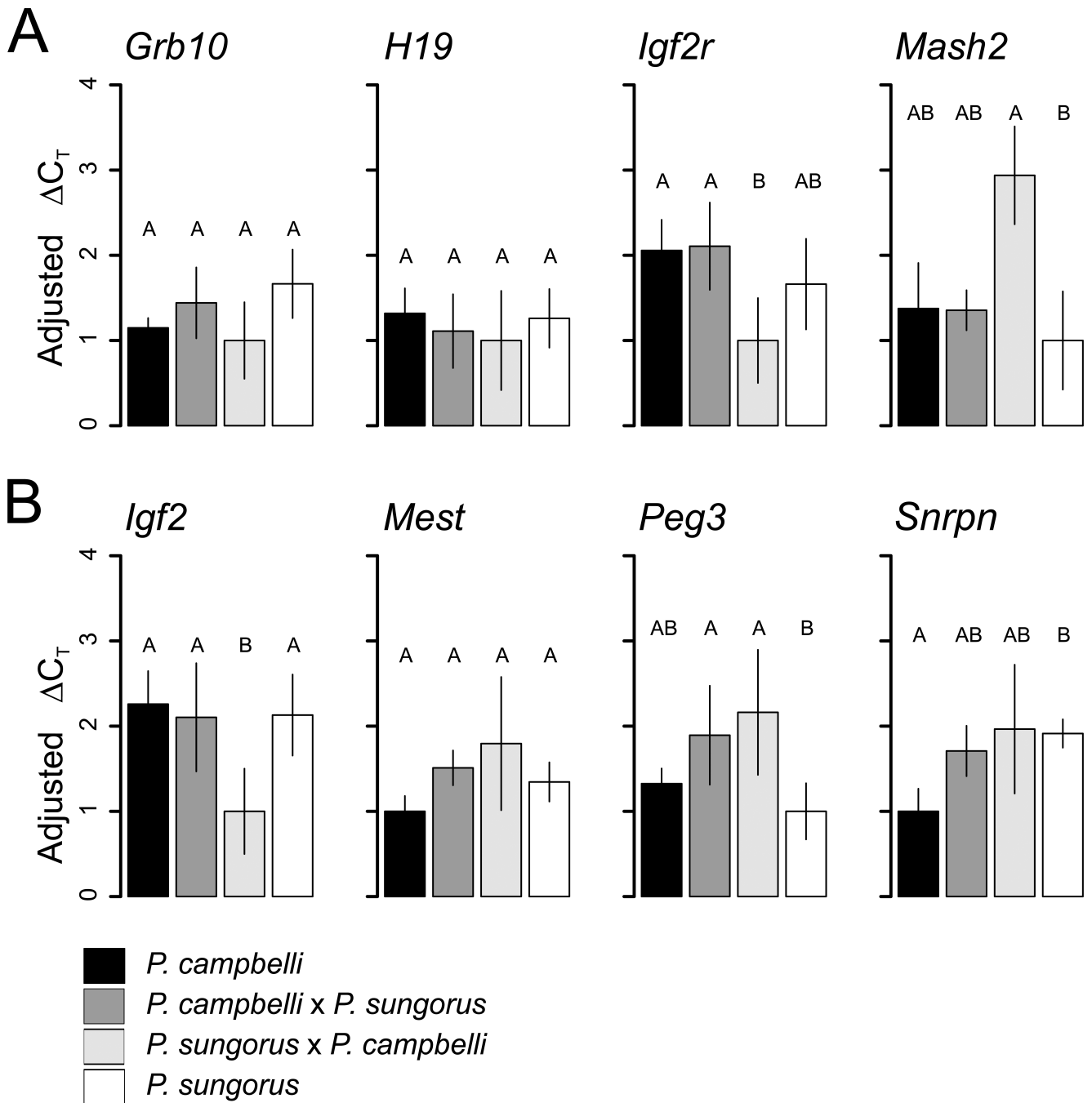


Figure 3.

Average weights in grams (± 2 SE) of hamsters at 80 days. Filled circles represent females, empty circles represent males, and sample sizes are given in parentheses. Hybrid cxs hamsters show reversed sexual dimorphism where males are smaller than females.

** $P < 0.001$, * $P < 0.05$, pairwise t -test, Bonferroni corrected $\alpha = 0.017$.

**Figure 4.**

Placental expression levels of candidate maternally (A) and paternally (B) expressed genes. Expression was normalized using the gene *Ywhaz* with the C_T method and subsequently adjusted to set the expression level of the lowest expressed cross type to one (± 2 SE). Bars represent the mean of each cross (n=6), significant differences were tested with an ANOVA ($P < 0.05$), and the lettered groupings were assigned based on a Tukey test.

Table 1

Mammal hybrids with observed growth effects. Phylogenetically independent crosses are in parentheses. Data are from Allen et al. (1993), Dawson (1965), Gray (1972), Sokolov (1993), and Zechner (1996). Details of the exact crosses can be found in Supplemental Table 1.

Order	Reciprocal crosses			Single crosses		
	Both hybrids larger than parent species	Parent of origin growth	Both hybrids smaller than parent species	Larger than parent species	Smaller than parent species	
Cetartiodactyla	5 (3)	1 (1)	-	4 (4)	1 (1)	
Carnivora	1 (1)	2 (2)	1 (1)	3 (3)	1 (1)	
Perissodactyla	1 (1)	1 (1)	-	3 (3)	1 (1)	
Primates	-	-	-	1 (1)	2 (2)	
Rodentia	1 (1)	6 (4)	-	6 (6)	-	
Total	8 (5)	10 (8)	1 (1)	17 (17)	5 (5)	

¹ $P < 0.001$, Fisher's exact test versus all other crosses, Bonferroni corrected $\alpha = 0.008$.

² $F_{3,43} = 12.811$, $P < 0.001$. Also significant in all pairwise Wilcoxon rank-sum tests, $P < 0.0073$, Bonferroni corrected $\alpha = 0.013$.

Late gestation litter size, sex ratio, and developmental defects of dwarf hamsters and their hybrids. Viable embryos are defined as those that were not molar conceptuses (Molar) or reabsorbing (Reab.), but may have edema. Only the viable embryos were used to calculate litter sizes and sex ratios. Significant values are in bold.

Table 2

Cross	Total	Viable Embryos				Molar	Reab.	% Male	Litter Size \pm se	Females / Litter \pm se	Males / Litter \pm se
		Litters	Females	Males	Unk. Sex						
<i>P. campbelli</i>	60	10	NA	NA	58	0	2	n.a.	5.8 \pm 0.7	n.a.	n.a.
<i>P. sungorus</i>	52	9	NA	NA	52	0	0	n.a.	5.8 \pm 0.5	n.a.	n.a.
<i>P. campbelli</i> \times <i>P. sungorus</i>	36	12	13	19	1	0	3	59.3	2.8\pm0.5 ²	1.1 \pm 0.3	1.6 \pm 0.3
<i>P. sungorus</i> \times <i>P. campbelli</i>	73	16	16	18	4	18 ¹	17 ¹	52.9	2.4\pm0.4 ²	1.0 \pm 0.2	1.1 \pm 0.2

¹ $P < 0.001$, Fisher's exact test versus all other cross types, Bonferroni-corrected $\alpha = 0.008$.

² $F_{3,43} = 12.811$, $P < 0.001$. Also significant in all pairwise Wilcoxon rank-sum tests, $P < 0.0073$, Bonferroni-corrected $\alpha = 0.013$.

Adult sex ratios in dwarf hamsters and their hybrids partitioned by litter. Sex ratio was tested with a chi-square test using the pooled sex ratio of the species (50.6% male) as the null expectation.

Table 3

Cross	Females	Males	Litters	% Male	Litter Size \pm se	Females/Litter \pm se	Males/Litter \pm se
<i>P. campbelli</i>	199	205	85	50.7	4.8 \pm 0.2	2.3 \pm 0.2	2.4 \pm 0.2
<i>P. sungorus</i>	229	226	77	49.7	5.9 \pm 0.2	3.0 \pm 0.2	2.9 \pm 0.2
<i>P. campbelli</i> \times <i>P. sungorus</i>	60	97	43	61.8 ¹	3.7\pm0.2 ²	1.4 \pm 0.2	2.3 \pm 0.2

¹ Chi-square test, $\chi^2=8.463$, $df=1$, $P<0.001$.

² $F_{2,202}=23.665$, $P<0.001$. Also significant in all pairwise Wilcoxon rank-sum tests, $P<0.004$, Bonferroni corrected $\alpha =0.025$.

Table 4

Hybrid expression of eight candidate imprinted genes in hamsters and deer mice. Predicted expression is based on the known *M.musculus* expression of these genes described in the Parent-of-Origin Effect Database (www.otago.ac.nz/IGC). At *Peg3*, one S×C male and one female show biallelic expression while all other hybrids show paternal expression. Similarly, *Snrpn* is biallelic in a single female, while the other five individuals showed imprinted expression. Deer mouse data are from Vrana (1998)

Gene name	Predicted expression	Hamster hybrid expression		Deer mouse hybrid expression	
		<i>P. campbelli</i> × <i>P. sungorus</i> (normal-sized)	<i>P. sungorus</i> × <i>P. campbelli</i> (large)	<i>P. maniculatus</i> × <i>P. polionotus</i> (small)	<i>P. polionotus</i> × <i>P. maniculatus</i> (large)
<i>Grb10</i>	Maternal	Biallelic	Biallelic	Biallelic	Biallelic
<i>H19</i>	Maternal	Maternal	Maternal	Maternal	Biallelic
<i>Igf2r</i>	Maternal	Biallelic	Biallelic	Biallelic	Maternal
<i>Mash2</i>	Maternal	Biallelic	Biallelic	Biallelic	Biallelic
<i>Igf2</i>	Paternal	Paternal	Paternal	Paternal	Paternal
<i>Mest</i>	Paternal	Not Diagnostic	Not Diagnostic	Paternal	Biallelic
<i>Peg3</i>	Paternal	Paternal	Paternal ¹	Paternal	Biallelic
<i>Snrpn</i>	Paternal	Paternal	Paternal ²	Paternal	Biallelic

¹One male and one female showed biallelic expression, four other hybrids showed unambiguous paternal expression.

²One female showed biallelic expression, five other individuals showed unambiguous paternal expression.