No evidence for large differences in genomic methylation between wild and hatchery steelhead (*Oncorhynchus mykiss*)

Michael S. Blouin, Virginie Thuillier, Becky Cooper, Vindhya Amarasinghe, Laura Cluzel, Hitoshi Araki, and Christoph Grunau

Abstract: When salmonid fish that have been raised in hatcheries spawn in the wild, they often produce fewer surviving adult offspring than wild fish. Recent data from steelhead (*Oncorhynchus mykiss*) in the Hood River (Oregon, USA) show that even one or two generations of hatchery culture can result in dramatic declines in fitness. Although intense domestication selection could cause such declines, it is worth considering alternative explanations. One possibility is heritable epigenetic changes induced by the hatchery environment. Here, we show, using methylation-sensitive amplified fragment length polymorphism, that hatchery and wild adult steelhead from the Hood River do not appear to differ substantially in overall levels of genomic methylation. Thus, although altered methylation of specific DNA sites or other epigenetic processes could still be important, the hatchery environment does not appear to cause a global hypo- or hypermethylation of the genome or create a large number of sites that are differentially methylated.

Résumé : Lorsque des poissons salmonidés élevés en pisciculture se reproduisent en nature, ils produisent souvent moins de rejetons survivants que les poissons sauvages. Des données récentes sur la truite arc-en-ciel anadrome (*Oncorhynchus mykiss*) dans la rivière Hood (Oregon, É.-U.) montrent que même une ou deux générations en pisciculture peuvent entraîner des réductions spectaculaires de fitness. Bien qu'une sélection intense reliée à la domestication puisse causer de tels déclins, il est important d'examiner les hypothèses de rechange. Parmi les possibilités, il y a les changements épigénétiques héritables causés par l'environnement de pisciculture. Nous montrons ici, à l'aide du polymorphisme de la longueur des fragments amplifiés sensible à la méthylation, que les truites arc-en-ciel anadromes de pisciculture et les truites sauvages de la Hood ne semblent pas différer de manière importante dans leurs niveaux globaux de méthylation génomique. Ainsi, alors que la méthylation altérée de certains sites d'ADN ou d'autres processus épigénétiques pourraient quand même être importants, le milieu de pisciculture ne semble causer ni une hypo-, ni une hyper-méthylation globale du génome, ni créer un nombre élevé de sites à méthylation distincte.

[Traduit par la Rédaction]

Introduction

Salmonid fish hatcheries are widely used to augment harvest and to support endangered wild populations (Williams 2006). Yet, a large body of evidence suggests that domesticated hatchery fish are genetically different from wild fish for a wide variety of adult and juvenile traits (Fleming and Petersson 2001) and that they often have much lower fitness than wild fish when they breed in the wild (Berejikian and Ford 2004; Araki et al. 2007*b*, 2008). What had not been appreciated before is how rapidly this fitness decline can occur. Araki et al. (2007*b*) showed that steelhead (*Oncorhynchus mykiss*) created using a wild parent and a first-generation hatchery parent have 55% the lifetime fitness of fish created in the same hatchery using two wild parents. Furthermore, a meta-analysis of estimates of lifetime fitness of several hatchery stocks relative to that of their wild ancestors suggests an average decline in fitness of 37% per generation during the first few generations of hatchery culture (Araki et al. 2007*b*). Unfortunately, the mechanisms by which captive rearing could cause such precipitous, heritable declines in fitness are not well understood.

The traditional explanations for why hatchery fish are less fit than wild fish in nature are mutation accumulation (relaxed natural selection; Lynch and O'Hely 2001) and do-

Received 30 April 2009. Accepted 6 October 2009. Published on the NRC Research Press Web site at cjfas.nrc.ca on 15 January 2010. J21178

Paper handled by Associate Editor Eric Taylor.

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mestication selection (Ford 2002). Relaxed natural selection is unlikely to cause dramatic fitness declines after only one or two generations unless salmon carry an enormous standing genetic load or have an unusually high mutation rate (Lynch et al. 1999; Araki et al. 2008). Araki et al. (2008) showed that if selection acts on a single trait, such rapid declines are indeed possible provided selection is very strong and that the heritability of the trait under selection is very high. If selection acts on multiple traits throughout the life cycle, then the parameter space (range of heritabilities and selection coefficients) required for driving such rapid fitness declines becomes less restrictive. Note that although high survival in the hatchery limits the opportunity for viability selection in the hatchery, there can still be strong selection later in the life cycle on phenotypic variation expressed during the hatchery phase of the life cycle (e.g., Reisenbichler et al. 2004).

Alternate mechanisms: epigenetic changes in DNA methylation

Although intense domestication selection could explain results such as those observed in Araki et al. (2007*b*), the declines were so rapid and precipitous that it is worth considering additional mechanisms. One intriguing possibility is heritable epigenetic changes induced by the hatchery environment. Epigenetic changes are mitotically or meiotically heritable changes in gene expression that result from modifications to the DNA without change in the DNA sequence (Richards 2006; Bird 2007; Jirtle and Skinner 2007). Epigenetic changes can be transmitted to the next generation (Richards 2006; Jirtle and Skinner 2007; Reik 2007). It is therefore conceivable that rearing in a hatchery environment during the early part of the salmon life cycle could cause epigenetic changes that eventually affect the fitness of those individuals and (or) their offspring.

The most well-studied epigenetic modification is methylation of cytosines, but chemical modification of histones and the production of transcription-regulating microRNAs are additional epigenetic mechanisms (Richards 2006; Bird 2007; Jirtle and Skinner 2007). Here, we focus on DNA methylation. Methylation is an important mechanism for control of gene expression in eukaryotes (Jost and Saluz 1992). In general, hypermethylation reduces transcription, while hypomethylation increases transcription. For example, methylation explains, in part, the approximately 1% of mammalian genes that show genomic imprinting (i.e., in which the maternally and paternally derived copies of a locus in an individual differ in expression: Reik and Walter 2001; Lewis and Reik 2006). A wide range of environmental stimuli such as diet, chemicals, or even social environment are known to produce methylation changes that can be passed on to subsequent generations (Anway et al. 2005; Jirtle and Skinner 2007; Reik 2007). For example, it has been known for a long time that raising plants in tissue culture generates many novel, heritable phenotypes and fitness declines. Genome-wide methylation changes appear to be associated with much of this variation (Kaeppler and Phillips 1993; Peraza-Echeverria et al. 2001).

Thus, it is reasonable to consider whether the novel environment of a fish hatchery can induce methylation changes that in turn induce variable phenotypes. Any number of environmental stimuli in a hatchery could induce methylation changes, including antibiotics, chemicals leaching from plastics or concrete, the artificial diet, or even the social environment (e.g., crowding levels). Stress per se could be the agent because cortisol levels are known to affect methylation patterns, as do a variety of other hormones (Vanyushin 2005).

The mutation accumulation hypothesis revisited

Methylation is also an important mechanism used by eukaryotic genomes to silence transposonable elements (Slotkin and Martienssen 2007). One consequence of the methylation changes that occur in plant tissue culture is genome-wide hypomethylation and the release of transposable elements (Hirochika et al. 1996; Cheng et al. 2006). Epigenetically controlled activation of transposable elements actually appears to be a response to stressful or novel environments in general (Capy et al. 2000; Slotkin and Martienssen 2007). Salmonid genomes are known to harbor a large variety and quantity of SINES, LINES, and other repetitive elements (Smit and Riggs 1995; Perez et al. 1999; Tafalla et al. 2006), and fish genomes are known to be heavily methylated (e.g., Macleod et al. 1999; Mhanni and McGowan 2004). Thus, the hatchery environment could cause an increase in the mutation rate via insertional mutagenesis and other position effects if it induced genome-wide hypomethylation, as occurs in plant tissue culture. Thus, the mutation accumulation hypothesis might not be so implausible after all, if hatcheries induce a higher mutation rate via epigenetic changes.

In summary, we know that (*i*) environmental variation can cause stable changes in methylation, (*ii*) such variation strongly affects gene expression, and thus the phenotype, (*iii*) these epigenetic changes, although reversible, are sometimes transmitted to the next generation, and (*iv*) epigenetic deregulation of transposable elements has the potential to increase deleterious mutation rates. Note that domestication selection and more exotic mechanisms such as enhanced mutation rates or epigenetic changes in gene expression are not mutually exclusive. Indeed, one can imagine such effects acting in concert to cause a severe drop in fitness in the second and later generations of hatchery culture. Thus, it seems reasonable to ask whether the novel environment of a hatchery induces epigenetic changes between hatchery and wild fish. Here, we test whether steelhead of wild and hatchery origin, derived from the same population, differ in overall levels of DNA methylation.

Materials and methods

Samples

We compared methylation patterns in wild, winter-run steelhead and in first-generation hatchery steelhead (those created in the hatchery using two wild parents) from the Hood River, Oregon (the same population studied by Araki et al. 2007*b*). Individuals were all caught as adults returning to the Powerdale dam (see Araki et al. (2007*a*, 2007*b*) for more details on these populations). We have an extensive pedigree for the fish in the Hood River and so were able to choose a set of each type of fish that were all unrelated. We chose only wild fish that we knew were the offspring of two wild parents in the previous generation.

Tests for differences in global levels of methylation

As a quick first look for gross differences in overall levels of methylation between hatchery and wild fish, we digested 3 μg of RNase-treated genomic DNA from each of three wild fish and three hatchery fish using the isoschizomers *Hpa*II and *Msp*I and then separated the fragments by electrophoresis through 0.8% agarose gels. Both enzymes recognize the restriction site CCGG but are differentially sensitive to methylation. *Hpa*II is inactivated in the presence of fully methylated inner C ($C^{5m}CGG$), whereas *Msp*I is active. Thus, *Msp*I cuts more frequently in methylated genomes than does *Hpa*II. We did a densitometric scan of digital photographs of the agarose gels and compared density distributions between the two types of fish. Density distributions appeared to be almost identical between the two types of fish (Fig. 1). This restriction digest test should be insensitive to all but the most dramatic, global changes in methylation. So we next used methylation-sensitive amplified fragment length polymorphism (msAFLP) (Xu et al. 2000; Keyte et al. 2006) on a larger sample of fish.

msAFLP assays the methylation status of individual restriction sites throughout the genome. Here, the procedure is like traditional AFLP (Vos et al. 1995) except that the frequently cutting enzyme is replaced by either *Hpa*II or *Msp*I. Each individual is run twice, once using *Hpa*II and once using *Msp*I. Then the AFLP patterns are compared between runs within each individual. Any bands that differ in presence–absence between *Hpa*II and *Msp*I runs on the same individual indicate the existence of at least one methylated site (Fig. 2). Note that a band present in both lanes indicates the existence of a fragment with unmethylated sites and matching selective bases next to the restriction sites on both ends. Any other combination (band present in *Msp*I but absent in *Hpa*II or vice versa) indicates the existence of a methylated site, but because a band is visible only if the selective bases on the AFLP primer match, one cannot conclude more than that (Fig. 2). One can imagine various scenarios in which a single methylation event could cause multiple bands to differ between the *Hpa*II and *Msp*I lanes (e.g.,Figs. 2*d* and 2*e*). However, the low probability of any new fragment also matching at the selective bases makes such multiple-band outcomes much less likely than single-band outcomes (as in Figs. 2*a*–2*c*). Averaged over many bands, testing whether hatchery and wild fish differ in the number or fraction of 0–1 + 1–0 bands should be a reasonable test of whether the number or fraction of methylated sites differs between types of fish. Note that because *Msp*I is insensitive to methylation, individuals that are genetically similar but differ in methylation at particular sites should show similar *Msp*I profiles but different *Hpa*II profiles. Thus, one could also test whether more bands than expected by chance alone differ between hatchery and wild fish for just the *Hpa*II profiles.

We performed msAFLP on 32 hatchery fish (12 females and 20 males) and 30 wild fish (18 females and 12 males) using *Msp*I, *Hpa*II, and four sets of selective primers. These samples were run in M.S. Blouin's laboratory at Oregon State University with three primer sets (sets 2–4, hereafter the "OSU data set"). We replicated part of the experiment on a subset of the fish (18 wild and 17 hatchery) with a different primer set (set 1) in C. Grunau's laboratory at the University of Perpignan (hereafter the "UP data set").

For each fish, we designated a band as 1–1 if it occurred in both the *Msp*I and *Hpa*II lanes, as 0–1 if present in the *Msp*I lane but not in the *Hpa*II lane, and as 1–0 if present in *Hpa*II but not in *Msp*I. For each primer set, we used two-tailed *t* tests to ask if the two types of fish differed in the following. (*i*) The average number of 1–0 bands (i.e., tests if fish types differ in the total number of bands revealed by the methylation-sensitive enzyme *Hpa*II). The logic here is that hypermethylated genomes should show fewer *Hpa*II bands. (*ii*) The average number of 1–1 bands. We expect to see fewer such bands in hypermethylated genomes. (*iii*) The average number of 0–1 plus 1–0 bands (i.e., the total number of methylation events detected; cf. Fig. 2). (*iv*) The fraction of bands that are 0–1 or 1–0 among all bands that are detected in each fish (i.e., (0–1 + 1–0)/(0–1 + 1–0 + 1–1) = fraction of bands showing evidence of methylation). We recognize that these four tests are not testing entirely independent hypotheses, but we view this as an exploratory analysis.

For the *Hpa*II profiles, we also tested whether each individual band differed in frequency between the two types of fish via a two-tailed Fisher's exact test using a 5% criterion. Here, we are asking if more bands differ in frequency between the two types of fish than expected by chance alone (i.e., 5% of them). The reasoning here is that types of fish could differ in methylation at many sites, but the direction might not be consistently towards hypo- or hypermethylation in one type of fish. In that case, the above *t* tests that focus on average levels of methylation would miss the pattern. Thus, we might still see an excess of bands that differ in frequency between hatchery and wild fish, even if there is no directionality to band presence or absence. To insure adequate power to reject the null hypothesis in each test, we did exact tests only on bands for which the frequency of the minor phenotype (band present or absent) in the entire set of fish was at least eight individuals (UP data set) or 10 individuals (OSU data set).

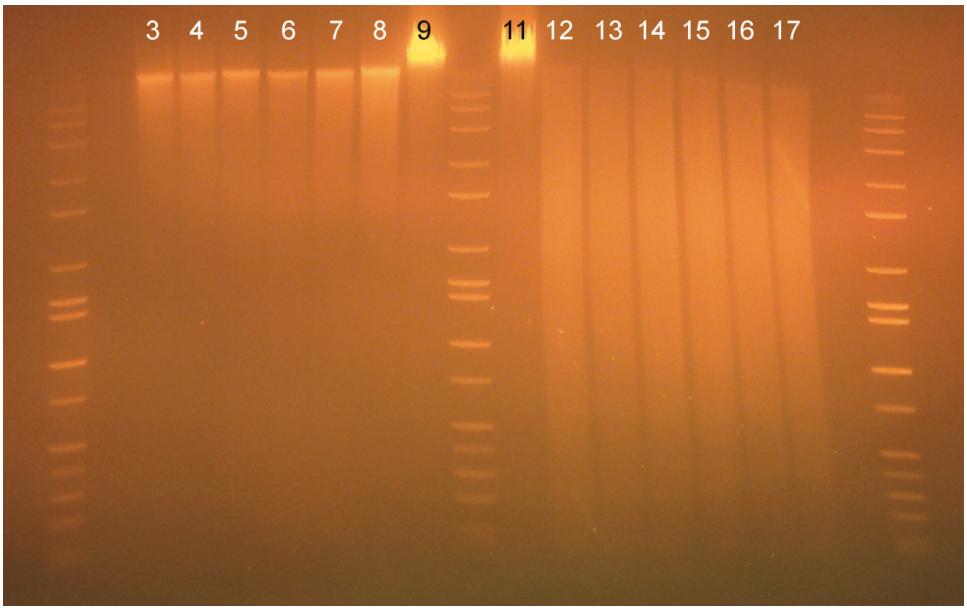
msAFLP detailed methods

We tried several selective primers and chose four sets that gave a large number of consistently scorable bands. The primer sets used were the *EcolR*I reverse primer R1-GACTGCGTACCAATTCCTG with one of the following *Hpa*II/*Msp*I forward primers: (set 1) F1-GATGAGTCTAGAACGGTCC), (set 2) F6-GATGAGTCTAGAACGGTGN, (set 3) F7-GATGAGTCTAGAACGGTAN, or (set 4) F9-GATGAGTCTAGAACGGTNN.

OSU data set

DNA was extracted with a standard phenol–chloroform extraction. Extractions were treated with RNase at a final concentration of 3 μg/μL. One microgram of DNA was doubly digested with either *EcoR*I and *Hpa*II or *EcoR*I and *Msp*I via the manufacturer's instructions (New England Biolabs). The samples then underwent ligation with adapter DNA fragments in the manner described in Xu et al. (2000). Amplification was done with primer sets 2–4 with the following cycling profile: 94 °C for 5 min, 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s; repeat steps 2–4 for 12 cycles; 94 °C for 30 s, 55 °C for 30 s, and 72 for 30 s; repeat steps 6–8 for 25 cycles; 72 °C for 7 min. The reverse primer of each set was labeled on the 5′ end with

Fig. 1. An 0.8% agarose gel showing restriction digest of 3 μg of genomic DNA using *Hpa*II (lanes 3–8) or *Msp*I (lanes 12–17). Results are shown for three hatchery adults (lanes 3–5 and 12–14) and three wild adults (lanes 6–8 and 15–17). Uncut genomic DNA is in lanes 9 and 11. The large shift in density distribution between *Hpa*II and *Msp*I lanes illustrates the heavy methylation present in fish genomes. The density distributions for both *Msp*I lanes and *Hpa*II lanes are very similar between hatchery and wild fish, indicating a lack of gross differences in overall levels of genome-wide methylation.



FAM dye. Resulting amplifications were run on an ABI 3100 capillary electrophoresis system to visualize the bands. Bands were called with a bin size of 1.0 bp and were corrected with visual inspection.

UP data set

Experimental conditions were as above with the following changes. Two hundred nanograms of DNA was used for the double digest, and polymerase chain reaction amplification was done as follows with primer set 1 and using 2 ng as template: 96 °C for 30 s; 14 cycles of 96 °C for 30 s, 65 °C for 30 s decreasing temperature by 0.7 °C every cycle, 72 °C for 1 min; 25 cycles: 96 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and 60 °C for 30 min. The reverse primer was labeled with WellRed D4-PA dye (Sigma) and amplicons were separated on a CEQ 8000 genetic analyzer (Beckman Coulter). Bands were called with a bin size of 1.2 bp and were corrected by visual inspection.

Experimental evaluation of sensitivity of the method

The literature on the msAFLP technique is unclear about how quantitative is the method (e.g., Yamamoto et al. 2001; Kageyama et al. 2008). In other words, can we use information on peak height, as well as on peak presence–absence, to infer variation between individuals in levels of methylation? By "levels of methylation", we mean the fraction of DNA molecules carrying a methylated copy of a particular nucleotide site. Therefore, we used CpG methyltransferase *M.Sss*I (NEB No. M0226) according to the instructions of the supplier to fully methylate all CpGs in two samples of steelhead DNA (individuals A and B). Then, we mixed each DNA sample with untreated DNA from the same fish to create five samples per fish that varied in the proportion of fully methylated DNA: 0%, 25%, 50%, 75%, and 100%. We then performed msAFLP on both sets of five samples using *Hpa*II primer set 1 and checked for bands that differed in peak height and (or) peak presence among samples. Each sample was run twice; only bands that were clearly present in both replicates were scored. Note that 0%, 25%, and so on are the mixture proportions, not the total fraction of the genome that is methylated. Taking into account the distribution of CpG methylation in vertebrates and a 5 mC content of roughly 1.5 mol% of total DNA (Varriale and Bernardi 2006), we assume that 75% of CpGs are methylated in salmon. So averaged over the entire genome, our five samples cover 75% (untreated), 81%, 87%, 94%, and 100% of CpG sites methylated. However, the distribution of methylated CpGs is not random in genomes. For example, CpG islands that occur 5′ of many genes often have little or no methylation, while intergenic regions tend to be heavily methylated. Thus, the comparison in this test is from "normal" levels up to 100% methylated CpGs, where "normal" values depend on the region of the genome being examined.

Results

Sensitivity of the method

We observed a total of 42 bands in individual A. The same 40 bands were present in the 0% (untreated) through 75% mixture DNA samples, with an additional two bands that were present in both replicates of only the 75% mixture sample (Table 1). All of these bands but three disappeared in the 100% methylated DNA sample. We observed a total 35 bands in individual B. We observed 32 bands in the 0% (untreated) sample and the appearance of three additional bands as the proportion of fully-methylated DNA increased (Table 1). All of individual B's bands disappeared in the 100% methylated sample. The appearance of a few additional bands in the

Fig. 2. Possible banding patterns given presence–absence of methylation at recognition sites. Vertical lines represent a DNA sequence with restriction sites represented as horizontal lines. Stars indicate a methylated CCGG cut site. Arrows indicate that the DNA sequence adjacent to a cut site matches the selective bases on the AFLP primer. "Eco" designates the *Eco*RI primer (fluorescently labeled). The other arrows are CCGG sites. To the right of each DNA sequence are hypothetical gel images for AFLP run using *Hpa*II or *Msp*I. (*a*) A single unmethylated fragment has the selective bases present at each end and so can be seen in both lanes. (*b*) Methylation of the CCGG site causes the band to disappear in the *Hpa*II lane. (*c*) A methylated but nonmatching site between the *Eco*RI site and a matching but unmethylated CCGG site causes the band to disappear in the *Msp*I lane. (*d* and *e*) Examples of how single methylation events could cause multiple bands to differ between the *Hpa*II and *Msp*I lanes. However, such multiple-band outcomes should be rare relative to outcomes illustrated in Figs. 2*a*–2*c* because selective bases must match for a band to be seen.

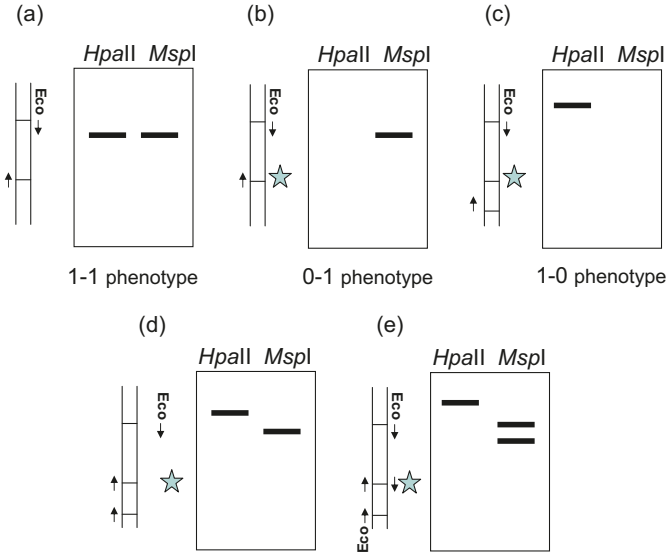


Table 1. Number of *Hpa*II-generated bands observed in the artificially methylated samples.

Mixture proportion (%)[a]	No. of bands in individual A	No. of bands in individual B
0	40	32
25	40	33
50	40	34
75	42	35
100	3	0

[a]Fraction of the sample that was artificially methylated using CpG methyltransferase (e.g., 25% means a 1:3 mix of treated and untreated DNA).

more heavily methylated samples probably resulted from partial methylation of sites that normally have 0% methylation. Therefore, although the technique can pick up large changes in methylation, it appears to be most sensitive to the presence–absence of methylated or unmethylated copies of a site and less sensitive to variation around intermediate levels of methylation. There was little variation in peak height among bands when they appeared. Thus, band presence–absence appears to be largely an all-or-nothing response.

Statistical analyses

OSU data set

We scored 165, 147, and 205 bands per primer set with *Hpa*II and 169, 179, and 158 bands per primer set with *Msp*I (for primer sets 2–4, respectively). About 60% percent of the bands in this sample of *O. mykiss* showed evidence of a methylated site (i.e., a 0–1 or 1–0 phenotype; Table 2), again reflecting the high level of methylation in salmonid genomes apparent in Fig. 1.

None of the *t* test results were significant except for one test on the absolute number of (0–1 + 1–0) bands using primer set 3, which indicated more such bands per wild fish (Table 2). However, that *P* value would not be significant if one adjusted for having done three independent tests of the hypothesis (three primer sets). Thus, we see no strong evidence that hatchery and wild fish differ in the absolute number of methylated (0–1 or 1–0) sites, in the fraction of total sites that are methylated ((0–1 + 1–0)/(0–1 + 1–0 + 1–1)), in the number of nonmethylated (1–1) sites, or in the number of sites identified by the methylation-sensitive enzyme (1–0).

When we tested each *Hpa*II peak for differences between hatchery and wild fish in band frequency (exact tests at 5% criterion), we observed no more significant results than expected by chance alone (5.2% of bands significant at $P = 0.05$; Table 3). Thus, there is no evidence that hatchery and wild fish differ in the frequencies of bands revealed by the methylation-sensitive enzyme beyond that expected by chance alone.

UP data set

We scored 141 bands for *Hpa*II and 159 for *Msp*I (primer set 1). Again, about 60% of bands showed evidence of a methylated site and none of the *t* test results were significant (Table 2). Three out of 43 (7%) exact tests done on the *Hpa*II bands gave significant results, again similar to results from the OSU data set (Table 3). Over all four data sets, the number of significant exact test results was 11/194 = 5.7% of the total number of bands tested, not significantly different from 5%.

Negative results bring up the question of power to detect an effect. For the exact tests, we only performed tests on bands for which the minor phenotype frequency was high enough to have power to detect a difference if one existed. That criterion left 194 bands that we tested for a significant difference in phenotype frequency between the two groups. We expected 9 or 10 (5%) significant bands by chance alone and observed 11 (5.7%). With 194 bands, we had 80% power to detect a difference if the true proportion of significant tests was 10% (estimated for a χ^2 test using SYSTAT 10; Systat Software Inc., Chicago, Illinois). Thus, if a large fraction of bands (say, 10% or more) really did differ in frequency between hatchery and wild fish, then we had a good chance of detecting that signal. For the *t* tests, one needs to decide what effect size would have been interesting to detect to estimate the power provided by our sample size (number of fish). To give an idea of what we could have detected, for each test, we also show the power that we had to detect an effect size (difference between means) of 10% of the smaller of the two means (Table 2) (estimated using SY-

Table 2. Average numbers of 1–0 bands (revealed by *Hpa*II only), 1–1 bands (no methylated sites), and absolute numbers and fraction of bands that were 1–0 or 0–1 (i.e., that showed evidence of a methylation event) in hatchery and wild steelhead.

Primer set	Dependent variable	Hatchery mean	Wild mean	*P*	Power[f]
OSU data set					
Set 2	1–1[a]	29.2 (3.4)[e]	29.4 (4.5)	0.821	0.78
	1–0[b]	9.8 (3.1)	10.4 (3.9)	0.603	0.18
	0–1 + 1–0[c]	37.75 (5.03)	39.2 (4.5)	0.228	0.83
	% 0–1 + 1–0[d]	56.25 (5.29)	57.14 (6.18)	0.545	0.95
	Number of fish	32	29		
	Sex ratio (F/M)	12/20	11/18		
Set 3	1–1	25.1 (4.4.)	25.6 (4.1)	0.677	0.55
	1–0	6.9 (3.1)	7.8 (2.6)	0.221	0.13
	0–1 + 1–0	42.1 (5.08)	45.53 (6.2)	0.028[g]	0.76
	% 0–1 + 1–0	62.6 (6.14)	64.23 (6.42)	0.338	0.94
	Number of fish	29	26		
	Sex ratio (F/M)	12/17	16/10		
Set 4	1–1	27.8 (5.7)	29.2 (5.0)	0.310	0.51
	1–0	13.3 (3.7)	12.9 (4.2)	0.617	0.25
	0–1 + 1–0	48.3 (6.8)	45.7 (5.87)	0.121	0.78
	% 0–1 + 1–0	63.38 (7.46)	61.17 (5.74)	0.197	0.93
	Number of fish	32	30		
	Sex ratio (F/M)	13/19	18/12		
UP data set					
Set 1	1–1	28.2 (4.45)	26.7 (6.00)	0.40	0.30
	1–0	42.1 (9.18)	37.9 (9.41)	0.19	0.21
	1–0 or 0–1	44.9 (5.74)	43.8 (5.01)	0.55	0.63
	% 1–0 or 0–1	61.41 (4.08)	62.4 (5.03)	0.52	0.96
	Number of fish	17	18		
	Sex ratio (F/M)	6/11	11/7		

Note: *P* values are for results of two-tailed *t* tests for difference in means between hatchery and wild fish. Sample sizes differ slightly between primer sets because we excluded any fish that did not give strong, reliably scored peaks throughout the entire size range examined.

[a]Present in both *Msp*I and *Hpa*II.

[b]Present only in *Hpa*II.

[c]Present in either *Msp*I or *Hpa*II.

[d]Percentage of total bands that are 0–1 or 1–0. No transformation was used because observations were mostly in the 50%–70% range and appeared normally distributed.

[e]Number in parentheses is the standard deviation.

[f]Power to detect a difference of 10% greater than the smaller mean assuming that both samples had the smaller sample size and the weighted average within-group variance.

[g]Significant difference.

Table 3. Exact tests for difference in *Hpa*II band frequency between types of fish.

Primer set	No. significant/no. tested[a]
OSU data set	
Set 2	1/52
Set 3	1/44
Set 4	6/55
All three OSU primer sets	8/151 (5.2%)
UP data set	
Set 1	3/43 (7.0%)
All four primer sets	11/194 (5.7%)

[a]Number of bands showing significant difference in frequency over the total number tested.

STAT 10; here, we used the weighted average within-group variance and assumed that both samples had the smaller of the two sample sizes). For that effect size, we had reasonable power (~0.55 to 0.95 for the OSU data set, slightly lower for the UP data set) for most tests except those on the number of *Hpa*II bands (owing to the much smaller number of bands in the *Hpa*II lanes). For that variable, differences of 20%–30% between means would have had to exist to have similar power.

Discussion

Results of our tests using mixtures of artificially methylated DNA suggested that band presence–absence is an all-or-nothing response. Thus, there appears to be little information about methylation levels to be extracted from peak heights. On the other hand, the all-or-nothing response gives us confidence in our ability to score bands as either present

or absent. That the technique is most sensitive for detecting changes in levels of methylation at sites that are near 100% methylation is also useful to know. For example, if a particular restriction site is normally methylated in all cells, and the hatchery environment caused some fraction of cells to not methylate that site, then we would expect to see a band appear or disappear. Similarly, if a site that is normally unmethylated in all cells is now sometimes methylated, we might again expect to see a band appear or disappear. On the other hand, if a site is normally methylated in, say 40% of cells, and the hatchery causes it to be methylated in 60% of cells, then we would not detect such a change.

Keeping in mind the above limitations of the technique, we saw no evidence for genome-wide variation in methylation levels between hatchery and wild fish. The *t* test comparisons showed no evidence of a large difference between types of fish in overall methylation level. Thus, for example, genome-wide hypomethylation, as occurs in tissue-cultured plants, is not obviously occurring in hatchery fish. Our exact tests on individual bands allowed for the possibility that fish types could differ in methylation level at many sites, but without an overall directionality (i.e., hatchery fish could be hypomethylated at some sites and hypermethylated at others, relative to wild fish). But only about 5% of the *Hpa*II bands differed in frequency between hatchery and wild fish, no more than expected by chance alone. Thus, we did not observe evidence for a large number of sites that are differentially methylated between hatchery and wild fish, in either direction.

Of course, these results do not rule out the existence of differences in methylation between hatchery and wild fish at particular genes. Thus, we have certainly not rejected hatchery-induced epigenetic effects as a cause of fitness declines in hatchery fish. We have simply shown that the hatchery does not induce large, global changes in methylation of the sort that could be detected using the methods employed here. Epigenetically controlled, differential expression of a handful of key genes could still contribute to differences in fitness between hatchery and wild fish. Of course, other epigenetic mechanisms such as histone modifications could also be involved but were not examined here. Searching for particular methylated sites would be better approached using methods other than those used here (e.g., BS-Seq; Cokus et al. 2008). An alternative approach to assessing the importance of differential methylation between hatchery and wild fish might be to first determine which genes are differentially expressed between them and then determine whether differential methylation explains differential expression of those particular genes.

Acknowledgements

We thank Michael Freitag for technical advice and comments on an earlier draft and the OSU Center for Genome Research and Biocomputing for assistance with fragment analysis.

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