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Our understanding is that over the last few years the number of Arctic grayling, *Thymallus arcticus*, in the spawning run from Red Rock Lakes into Red Rock Creek has been declining. If so, this could decrease the number of spawning fish, which could decrease effective population size (N_e), and eventually the amount of genetic variation in the population. N_e determines the rate at which random processes (genetic drift) remove genetic variation from populations. As N_e progressively decreases over time the amount and rate at which genetic variation is removed from the population is expected to increase and eventually the loss of genetic variation may be so severe that it compromises population viability. N_e is often less than the census population size because it does not consider immature individuals and it corrects for deviations from a 50:50 sex ratio among the parents and a Poisson distribution with a mean of two for number of offspring produced among parents. A problem with N_e is that it is almost invariably extremely difficult, if not impossible, to estimate in natural populations. Thus, a more estimable parameter referred to as the effective number of breeders (N_b) is often used (Waples and Do 2008). N_b basically estimates the number of parents that effectively produced a cohort.

Obviously if the perceived population decline of grayling in Red Rock Lakes is real, then rectifying the causes is warranted from a number of perspectives. In order to prevent and reverse the population decline, one ideally would like to determine its causes and implement remedial actions. In many situations, this may not be possible for a number of years. In these cases, 'band aid' technology is often employed until the causes of the decline can be identified and rectified. An interim measure for Red Rock Creek grayling could be to temporarily supplement the population with fish captured from it. If properly employed, this could ameliorate the negative genetic and demographic effects of the decline. This measure would only be effective if the causes of the decline occur sometime during the early life stage of the fish. From a genetics perspective, a possible drawback of a supplementation program is that the fish used for it may effectively be produced from a small number of individuals. If this is repeated for a number of generations, then the supplementation program could hasten the loss of genetic variation from the population and eventually do more harm than good.

In the spring of 2016, 38 grayling were captured from Red Rock Creek and were spawned, generally using a 2 X 2 matrix, to produce fish in order to supplement the population and to begin attempting to establish a population in what is believed to be fishless Handkerchief Lake. A 2 X 2 spawning matrix crosses two males with the same two females by roughly dividing the female's eggs into what appear to volumetrically (numerically) be two equal lots of eggs. Each lot is then fertilized using milt from only one of the males but, the two lots of eggs from a female are fertilized using milt from two different males. This is then repeated using the eggs from the other female and the milt from the same two males. This of course could not be carried out with all individuals because some of the females had too few eggs to produce two lots, some of

the males could not be used to fertilize two females, and some fish did not have adequately mature gametes.

While 38 grayling were used as parents, fin clips from 9 of the parents were misplaced. Thus, we were provided with fin clips from only 29 (14 males and 15 females) of the parents (76%) for genetic analysis. DNA was extracted from the fin clips taken from the parents and a random sample of fish collected from Red Rock Creek in 2016. DNA was also extracted from a random sample of 100 fry produced from the 2016 parents and maintained at the Somers State Hatchery. The DNA extracted from these individuals was used to determine each fish's genotype at 12 microsatellite loci. From these data, amounts of genetic variation in the samples were estimated by calculating average expected heterozygosity (H_e; henceforth heterozygosity), average number of alleles per locus (A), and allelic richness (A_r) which is simply the average number of alleles per locus standardized to the smallest sample size. The data from the 2016 random sample allowed comparison to previously collected samples from Red Rock Creek or Lakes to determine whether or not amounts of genetic variation differed among them. The data also allowed a determination of the family structure of the 2016 progeny and whether or not, from a genetics perspective, the progeny appeared to be a suitable source of fish for supplementing the population and to stock into Handkerchief Lake.

We have comparable microsatellite data obtained from random samples of Red Rock Creek or Lakes grayling collected in 2007, 2008, 2014, 2015, and 2016. Amounts of genetic variation, as estimated by H_e, among these samples appears to be declining. This reduction in heterozygosity mainly contrasted the 2007, 2008, and 2014 samples (average H_e=0.760, old samples) to the 2015 and 2016 samples (average H_e=0.737, new samples; Table 1). The difference in the amount of heterozygosity lost between the new and old samples was not exceptionally large (3.0%) but, bear in mind this rate of loss corresponds to an effective population size of about 17 per generation. If this N_e, persists it will result in a 24% overall loss of heterozygosity after 30 years or about eight to ten generations of essentially random mating. At this point, the reduction in the amount of genetic diversity in the population is almost certainly to at least begin to have detrimental effects on population viability.

Most of the fish in the 2014-2016 samples were aged using scales. This allowed placement of the individuals into year classes and estimation of levels of genetic variation in these year classes. The fish in the samples were mainly assigned to the 2011-2013 year classes. Compared to all the other samples, the fish in the 2011-2013 samples had the lowest amounts of heterozygosity observed.

Arctic grayling begin spawning at age 3 so it would not have been until 2014 that individuals from the 2011 year class began spawning. In 2015, individuals from both the 2011 and 2012 year classes would have been sexually mature. Finally, in 2016 individuals from the 2011-2013 year classes would have been mature.

We suspect the low genetic variation observed in the 2011-2013 year classes may at least be partially responsible for the reduced genetic variation observed in the 2015 and 2016 samples. If we assume that greater contribution from the 2011-2013 year classes to a mixed-aged sample increases the genetic effects these year classes will have on the sample, we expect the 2011-2013 year classes to have the greatest negative effect on genetic diversity in the 2015 and 2016 samples. That is, the 2014 sample contained age 3 and 4 fish with the age 3 fish coming only from the 2011 year class. Thus, the reduced genetic variation in the 2011-2013 year classes probably did not have that much of an effect on the estimate of H_e in the 2014 sample. In contrast, the 2015 sample contained fish aged 3 through 7, with the 3 and 4 year old fish coming from the 2011 and 2012 year classes. Now the reduced H_e in the 2011-2012 year classes may have started to detectably effect H_e in the samples. Finally, the 2016 sample contained fish aged 2 through 5 with the 2011 (age 5), 2012 (age 4), and 2013 (age 3) year classes having a genetic contribution to the sample.

Another estimator of amounts of genetic variation in samples (populations) is the average number of alleles per locus (A). In fact, this estimate is more sensitive to a reduction in amounts of genetic variation than H_e because H_e is not sensitive to a loss of low frequency alleles while A is. In the Red Rock Creek population, A appears to have decreased from a mean of 13.8 among the 2007, 2008, and 2014 samples to a mean of 12.7 between the 2015 and 2016 samples (Table 1). This represents about an 8.1% decrease in the average number of alleles per locus in the Red Rock population from 2007-2016. Thus, these data add additional support to the premise that the Red Rock Lakes population is numerically declining and this decline is detectably reducing levels of genetic variation in it.

A drawback of using A as an estimate of the amount of genetic variation in samples is that to a point it is affected by sample size. In other words, the bigger the sample size the more likely one is to encounter low frequency alleles and mathematically not biologically increase A. In order to remove this problem from the data, for comparative purposes one often compares allelic richness (A_r) among samples. A_r is simply the average number of alleles per locus standardized to the smallest sample size in the data set (N = 56 for the comparison of mixed-aged samples) so the effect that disparity in sample size has on A is largely removed. When this is done with our data, the results are basically the same as those obtained using A. There was a 7.1% decline in A_r between the mean of the 2015-2016 samples compared to the mean of 2007-2014 samples. Overall, therefore, the genetic data add support to the hypothesis that levels of genetic variation, regardless of how it is estimated, are decreasing in the Red Rock Lakes population. Thus, it becomes somewhat important to attempt to determine the cause of the demographic and genetic declines and implement remedial actions.

We now address the issue of whether or not the Artic grayling produced by spawning fish from Red Rock Creek in 2016 constitute a suitable source to supplement Red Rock Creek and to attempt to re-establish fish into what now is considered to be fishless Handkerchief Lake. Recall, the 2016 Arctic grayling were produced from at most 38 adults using largely a 2 X 2 spawning matrix and thus a maximum of 76 full-sib families could have been produced. Based on a random sample of 100 fry from these parents, the fry had very reduced levels of genetic variation compared to what was observed in the 2015-2016 samples (Table 1). We used the program LDNe of Waples and Do (2008) to estimate the effective number of parents (N_b) that produced the 2016 fry year class. Although 38 fish were spawned to produce the fry, N_b was only 18.9 (CI=16.8-21.3). This was substantially smaller than similar estimates for other year classes. For example, the estimate of N_b for the 2011 year class at age 4 or 5 was 177.8 (CI=113.0-375.5), the 2012 year class at age 3 or 4 was 126.3 (CI=83.7-236.7), and the 2013 year class at age 3 was 678.7 (CI=214.0-Infinity). We are not overly surprised by this result because only 38 fish were used via anthropogenic spawning to produce the 2016 year class (hence the 2016 year class) and probably not all of these individuals contributed progeny to the 2016 year class.

Because of the small number of parents used to produce the 2016 year class we also determined the relationship of all individuals in the sample of the 2016 fry. The results suggested that individuals in the sample came from 25 not 76 full-sib families. The number of fish per full-sib family in the sample was quite variable ranging from 1 to 15. Out of the 25 families, 20 contributed 1-4 fry to the sample, 1 family contributed 9 fry, 2 families contributed 10 fry each, 1 family contributed 11 fry, and 1 family contributed 15 fry (Figure 1). The last five families (ten adults), therefore, produced about 55% of the fish in the 2016 year class sample. Thus, in conjunction with the relatively small number of parents that produced the 2016 year class it appears that there is also high variance among families for the number of fish contributed to the 2016 year class of Arctic grayling. This variance in reproductive success among families further acts to reduce the effective number of spawners that produced the 2016 fry sample to about 19. Almost invariably, half sibling assignments are less reliable with current sibship reconstruction methods than full sibling analysis. The reliability of half sibling assignments, however, increases as family size increases. Thus, in our case the half-sib assignments for the five largest families are likely to be reliable enough for our purposes. The largest full-

sib family (15 offspring in the sample) and one of the third largest full-sib families (10 offspring in the sample) appear to be half siblings that share the same mother (based on reconstruction of parental genotypes based on the full-sib assignments). Thus, this one female (h47, from the 2013 cohort sampled in 2016) produced 25 of the 100 offspring sampled, again pointing to high variance in reproductive success among the fish anthropogenically spawned to create the 2016 hatchery population.

The small number of individuals spawned to produce the 2016 hatchery year class was exacerbated by the high variance in reproductive success among the parents resulting in very low N_b and levels of genetic variation in the fish. Because of the very low levels of genetic variation in the 2016 hatchery year class and the declining trend in levels of genetic variation in the Red Rock Creek population, introducing fish from the 2016 hatchery year class into the Red Rock Creek population could have highly negative consequences. "Supplementation" of a natural population demonstrating a declining trend in amounts of genetic diversity with fish produced from it, but with even lower levels of genetic diversity because of low N_b, could very well serve to further reduce the effective size of the natural population, especially if supplementation is done over a number of years. Supplementation with the 2016 hatchery year class could hasten the erosion of genetic diversity in the supplemented population.

Handkerchief Lake is believed to be devoid of salmonid fishes. From the available data, the 2016 hatchery year class of Red Rock grayling could be used as an initial effort to establish a grayling population in the lake. You have indicated the primary purpose for the introduction is to have a "back-up" for the Centennial Valley native populations, i.e. Red Rock and Elk lakes. In the following considerations, we assume that you wish to maintain the newly founded Handkerchief population as a "genetic reserve", ruling out the use of fish from other sources for supplementation. This is a difficult decision. We lay out three alternatives below. All of the alternatives involve the use of progeny from a limited set of parents. The decision that must be made is whether or not to attempt to equalize the contribution from this limited set of parents. That is, should all the fish be used to found Handkerchief Lake? Or should an attempt be made to equalize family contribution. This would entail taking measures to equalize the contribution of hatchery-reared families prior to introducing them to the lake. For the 2016 Red Rock hatchery year class, this would require genotyping individuals, assigning them to full-sib families based on their genotypes, and determining membership for a subset of fish to be stocked based on full-sib assignments. We lay out three alternatives and take into account the influence of each on the number of fish outplanted to Handkerchief Lake, the resulting N_b, the expected proportional loss in heterozygosity in the first cohort produced, and the expected cost.

Alternative A – Equalize family contribution

We used the distribution of full-sibling families from the 100 fry sample to provide guidance for genotyping efforts to determine how many fry should be genotyped and which ones should be stocked into Handkerchief Lake (Table 2). This was basically a fairly crude cost benefit analysis. The first two columns of Table 2 show the number of full-sibling families (N = 25) and the estimated size of those families in the random sample of 100 fry (range 1 – 15 sibs). The proportional composition of full-sibling families of the 100 fry sample is shown in the third column. In subsequent columns, we show the expected number of fish from each full-sibling family in a sample of 3,000, 2,000, 1,500, 1,000, and 500 fish to be genotyped. In addition, we show numbers of individuals per family that could be stocked using a threshold of no more than 40 per family. This threshold of 40 means that at most 1,000 individuals will be stocked and in the large families many fish identified will not be stocked and in the smaller families often less than 40 will be available for stocking. These deficits will not be "made up" by adding additional fish from the larger families. The 40 fish threshold could be changed, but a larger threshold will lead to increased variance in full-sibling family contribution (and a reduced N_b), while a smaller threshold would lead to fewer stocked fish. We assume that costs exceeding $40,000 become prohibitive. This means that this alternative would require the analysis of 1,000 fish for potential stocking, stocking 650 fish, and obtaining an expected N_b of 44, and an expected

proportional loss of He of 0.011 in the first cohort produced. An additional cost for this alternative, but one not considered here, is the need to tag individual fish once they are fin clipped, so that genotype-based full-sib family membership can be associated with each genotyped fish.

Alternative B – Genotype offspring but attempt to increase N_c relative to Alternative A

Since 650 outplanted fish might not be enough, even if we expect high survival because of the fishless condition of the lake due to poisoning. Therefore, we propose an alternative scenario that involves the following (Table 3): (1) 1,000 fry from the 2016 year-class are randomly selected and outplanted to Handkerchief Lake without being genotyped (Table 3, column 4). (2) A second 1,000 fry are genotyped (and tagged so each fish can be associated with its genotype) to assign full-sibling family membership. (3) Individuals assigned to the top 5 largest full-sib families are culled, the remainder of fish are outplanted. This leads to another 450 fish that can be added to Handkerchief Lake (Table 3, column 6), for a total of 1,450 available for outplanting (Table 3, column 7). Alternative B would have the same cost as Alternative A ($40,000), but would allow the outplanting of an additional 800 fish. N_b would be lower (38.6 vs. 44), and the expected proportional loss of heterozygosity in the first cohort produced would be greater (0.013 vs. 0.011).

Alternative C – No genotyping, outplant all hatchery reared fish

Scenarios A and B must be weighted against the option of outplanting all of the 2016 year class to Handkerchief Lake without any attempt to genotype offspring and equalize family contribution. Alternative C is shown in Table 2, column 4. All 3,000 fish are outplanted, so N_c is maximized. Variance in family contribution leads to a minimum N_b (26.9) and a maximum expected proportional loss of heterozygosity in the first cohort produced (0.019). This option incurs no genotyping costs and no costs associated with tagging fish while they are in the hatchery.

We recommend Alternative C. None of the alternatives allows us to avoid the fact that there were few breeders represented in the 2016 hatchery year-class. Attempts to equalize family contribution (Alternatives A and B), in our opinion, do not do enough to minimize genetic effects or get around the fact that there are few adults contributing to this initial founding population to warrant the expense. Under any of the alternatives, the worst case scenario is that the five dominant families in the 2016 year class continue to remain numerically dominant in the Handkerchief Lake. However, this could happen even if we attempt to equalize family contributions. You have also indicated that there should be adequate spawning habitat in the tributary to Handkerchief Lake, which will hopefully allow as many possible spawners during natural reproduction when it occurs. However, keep in mind that even if representatives from every full-sib family contribute to the first year-class produced in Handkerchief Lake, the total number of families (estimated at 25) is still quite small. We therefore strongly suggest that, after the entire 2016 hatchery year-class is added to Handkerchief Lake, every attempt is made to add more fish to this lake as soon as possible (from either Elk Lake or Red Rock Creek), that is, as soon as it is deemed an acceptable risk to either of these populations. In the mean time, genetic monitoring of the newly founded Handkerchief population should be implemented to test for evidence of loss of genetic diversity and numerical dominance of the five top families. If more genetic material cannot be added to Handkerchief Lake prior to the first spawning of the 2016 year-class, there is a real possibility that the new population will lose so much genetic diversity that it would be difficult to continue to call it a genetic reserve, and genetic monitoring would allow us to assess this. Finally, your idea of preventing spawning in Handkerchief Lake in 2018 might be a good one (to help prevent dominance by these 2016 year-class fish, should addition of more fish prove unfeasible by 2018), but we suggest that consideration of this option wait to see if what happens in the next year.

This report was prepared by Andrew Whiteley and Robb Leary

References Cited

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Waples, R. S., and C. Do. 2008. LDNE: a program for estimating effective population size from data on linkage disequilibrium. Molecular Ecology Resources **8**:753-756.

Waples, R. S., and R. K. Waples. 2011. Inbreeding effective population size and parentage analysis without parents. Molecular Ecology Resources **11**:162-171.

Table 1. Summary of results for Arctic grayling from Red Rock Creek and 2016 hatchery-reared offspring. Shown are sample size (N), expected heterozygosity (H_e), mean number of alleles (A), mean allelic richness (Ar), the estimated number of full-sibling families, the mean estimated full-sibling family size, and effective number of breeders (N_b), with 95% confidence intervals. Fish were captured from Red Rock Creek in 2014 through 2016. We examined these mixed-age classes without assigning fish to cohort to obtain estimates of genetic diversity ('Wild fish –mixed age sample by year'). Wild-caught fish were assigned to cohorts with scale-derived ages and these cohorts were examined separately ('Wild fish – assigned to cohort'). Finally, the hatchery-reared 2016 cohort was examined separately ('Hatchery-reared fry'). Estimates of full-sib family structure and N_b were only conducted using the 2011-2013 scale age determined cohorts and the 2016 fry.

Sample	N	H_e	A	Ar	Numb. full-sib fam	Mean full-sib fam size	N_b
Wild fish - mixed age by sample year							
2007	228	0.764	14.7	12.7	NA	NA	NA
2008	100	0.754	13.9	12.6	NA	NA	NA
2014	56	0.761	12.8	12.7	NA	NA	NA
2015	81	0.735	12.5	11.8	NA	NA	NA
2016	98	0.739	12.8	11.7	NA	NA	NA
Wild fish - assigned to cohort							
2011	56	0.725	11.5	11.0	49	1.1	177.8 (113.0-375.5)
2012	46	0.738	11.0	11.0	41	1.1	126.3 (83.7-236.7)
2013	49	0.730	11.7	11.5	44	1.1	678.7 (214.0-INF)
Hatchery-reared fry (2016 cohort)							
2016	100	0.703	10.5	10.5	25	4	18.9 (16.8-21.3)

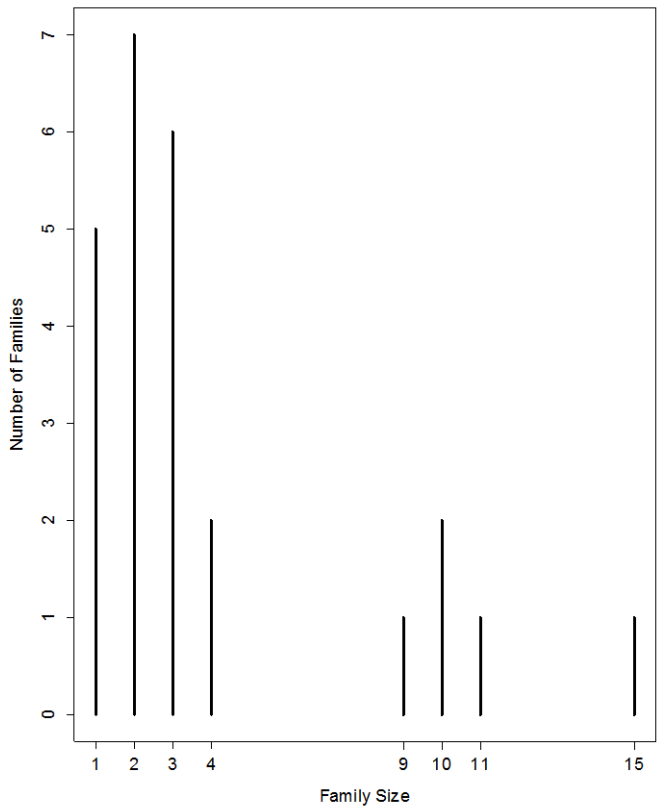

Figure 1. Histogram of full-sibling family sizes for the randomly sampled 100 hatchery-reared fry from the 2016 cohort. 55 of 100 offspring assigned to the five largest full-sib families.

Table 2. Estimation of genotyping effort needed to balance the need to equalize family contribution, cost, and number of fish stocked into Handkerchief Lake (Alternative A and C). The first two columns show the number of full-sibling families and the estimated size of those families from the 100-fry sample. The proportional composition of full-sibling families of the 100 fry sample is shown in the third column. In subsequent columns, we show the full-sibling family sizes we expect to observe from random subsamples of 3,000, 2,000, 1,500, 1,000, and 500 fish to be genotyped. In addition, we show family size distributions once a threshold family size of 40 fish is employed. This threshold family size would allow a balance between maximizing the number of fish stocked and attempting to equalize family contribution to the founding population. The number of fish stocked per genotyping scenario and the associated N_b, the expected proportional loss of heterozygosity in the next cohort produced for a given N_b, and estimated cost is shown at the bottom of the table. N_b was calculated according to Waples & Waples (2011). Expected loss of heterozygosity was calculated as $1/(2* N_b)$. Cost is based on the number of fish genotyped at $40/fish.

			Genotype 3000		Genotype 2000		Genotype 1500		Genotype 1000		Genotype 500	
Full-Sib Family Number	Family Size	Family size proportion	Expected number per family	40 fish threshold	Expected number per family	40 fish threshold	Expected number per family	40 fish threshold	Expected number per family	40 fish threshold	Expected number per family	40 fish threshold
1	15	0.15	450	40	300	40	225	40	150	40	75	40
2	11	0.11	330	40	220	40	165	40	110	40	55	40
3	10	0.1	300	40	200	40	150	40	100	40	50	40
4	10	0.1	300	40	200	40	150	40	100	40	50	40
5	9	0.09	270	40	180	40	135	40	90	40	45	40
6	4	0.04	120	40	80	40	60	40	40	40	20	20
7	4	0.04	120	40	80	40	60	40	40	40	20	20
8	3	0.03	90	40	60	40	45	40	30	30	15	15
9	3	0.03	90	40	60	40	45	40	30	30	15	15
10	3	0.03	90	40	60	40	45	40	30	30	15	15
11	3	0.03	90	40	60	40	45	40	30	30	15	15
12	3	0.03	90	40	60	40	45	40	30	30	15	15
13	3	0.03	90	40	60	40	45	40	30	30	15	15
14	2	0.02	60	40	40	40	30	30	20	20	10	10
15	2	0.02	60	40	40	40	30	30	20	20	10	10
16	2	0.02	60	40	40	40	30	30	20	20	10	10
17	2	0.02	60	40	40	40	30	30	20	20	10	10
18	2	0.02	60	40	40	40	30	30	20	20	10	10
19	2	0.02	60	40	40	40	30	30	20	20	10	10
20	2	0.02	60	40	40	40	30	30	20	20	10	10
21	1	0.01	30	30	20	20	15	15	10	10	5	5
22	1	0.01	30	30	20	20	15	15	10	10	5	5
23	1	0.01	30	30	20	20	15	15	10	10	5	5
24	1	0.01	30	30	20	20	15	15	10	10	5	5
25	1	0.01	30	30	20	20	15	15	10	10	5	5
Number stocked (sum)			3000	950		900		805		650		425
N_b			26.9	50.7		48.9		47.2		43.8		34.2
Expected proportional loss of heterozygosity in next cohort produced			0.019	0.010		0.010		0.011		0.011		0.015
Cost			$0	$120,000		$80,000		$60,000		$40,000		$20,000

Table 3. Calculations for Alternative B. For this option, 1,000 fish are released into Handkerchief lake without any genotyping or sibship assignment. The expected number per family for these 1000 fish is shown in the fourth column. An additional 1,000 fish are genotyped and assigned to full-sibling families. All individuals from the top 5 families are removed and the remainder of the fish (N = 450) are released in Handkerchief lake. The final column shows the combined expected number of fish released per family. The total number of fish stocked is 1450. Also shown is the estimated N_b, expected proportional loss of heterozygosity in the next cohort produced for this N_b, and estimated cost. N_b was calculated according to Waples & Waples (2011). Expected loss of heterozygosity was calculated as $1/(2* N_b)$. Cost is based on the number of fish genotyped at $40/fish.

			Release 1000	+ Genotype additional 1000, remove 5 largest families	=	Total
Full-Sib Family Number	Family Size	Family size proportion	Expected number per family	Expected number per family	Remove 5 largest families	Expected number released per family
1	15	0.15	150	150	0	150
2	11	0.11	110	110	0	110
3	10	0.1	100	100	0	100
4	10	0.1	100	100	0	100
5	9	0.09	90	90	0	90
6	4	0.04	40	40	40	80
7	4	0.04	40	40	40	80
8	3	0.03	30	30	30	60
9	3	0.03	30	30	30	60
10	3	0.03	30	30	30	60
11	3	0.03	30	30	30	60
12	3	0.03	30	30	30	60
13	3	0.03	30	30	30	60
14	2	0.02	20	20	20	40
15	2	0.02	20	20	20	40
16	2	0.02	20	20	20	40
17	2	0.02	20	20	20	40
18	2	0.02	20	20	20	40
19	2	0.02	20	20	20	40
20	2	0.02	20	20	20	40
21	1	0.01	10	10	10	20
22	1	0.01	10	10	10	20
23	1	0.01	10	10	10	20
24	1	0.01	10	10	10	20
25	1	0.01	10	10	10	20
Number stocked (sum)			1000		450	1450
N_b						38.6
Expected proportional loss of heterozygosity in next cohort produced						0.013
Cost						$40,000