# A COMPARISON OF GENETIC VARIATION BETWEEN BLACK-CROWNED NIGHT HERON (*Nycticorax nycticorax*) POPULATIONS FROM CONTAMINATED AND REFERENCE SITES

A Thesis

by

DANIELLE SUMMER BERNARD

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2005

Major Subject: Wildlife and Fisheries Sciences

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Approved by:

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December 2005

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## ABSTRACT

A Comparison of Genetic Variation between Black-crowned Night Heron (*Nycticorax nycticorax*) Populations From Contaminated and Reference Sites.

(December 2005)

Danielle Summer Bernard, B.S., Long Island University

Chair of Advisory Committee: Dr. Keith A. Arnold

I examined genetic variation for two populations of Black-crowned Night Herons using a 467 base pair region of the mitochondrial DNA. One population inhabits an environment highly impacted by industrial waste, heavy metals, and urbanization; while the other, a reference population, comes from a contaminant-free area. I observed a total of 10 haplotypes, three of which the two populations share. One individual from the contaminated site was ostensibly heteroplasmic. I found no evidence of significant genetic differentiation between the two populations. Coalescent simulation results provided evidence that both populations have undergone or are currently undergoing population expansion. The results of the biological marker I developed showed a high diversity for the ND-6 gene, making it a useful biomarker of population effects.

## DEDICATION

I dedicate this research to my loving family. They stood by me through all the ups and downs of this project and gave me the encouragement I needed to continue onward. I would also like to give my sister a special acknowledgement for coming to the lab with me late at night to keep me company and to keep me sane. I love you all.

## ACKNOWLEDGEMENTS

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I would also like to acknowledge Dr. John Bickham's lab for providing me with this opportunity, as well as aiding in my completing of this research.

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# INTRODUCTION

Black-crowned Night Herons (BCNH) *Nycticorax nycticorax,* a nocturnal species, inhabit wetland areas throughout most of the contiguous United States (Golden et al. 2003). BCNH have long been considered useful biological indicators of the effects of environmental contamination (Custer et al. 1994). Reasons for studying this species include their ability to bioaccumulate toxins because they feed high on the food chain, their generalized feeding habits, their colonial nesting habits, and their broad geographic distribution and migration patterns (Custer et al. 1991, 1994; Erwin et al. 1991; Rattner et al. 1993).

Despite the ability of these birds to bioaccumulate contaminants and utility as a sentinel species, little is known of their population genetics. A previous study of BCNH investigated the Cytochrome *b* (Cyt *b*) gene of the mitochondrial DNA (mtDNA; Dahl et al. 2001). Reasons for investigating mtDNA include 1) strict maternal inheritance of the marker; and 2) the mitochondria often are the target affected by contamination. Utilizing mtDNA as a marker, studies comparing contaminated and noncontaminated populations have demonstrated decreased genetic diversity at contaminated sites, increased mutation rates at contaminated sites, and evidence of ecological sinks (Bickham et al. 2000). However, the only previous investigation of BCNH failed to reveal sufficient genetic variability to adequately test for genetic effects (Dahl et al. 2001).

In order to detect the potential genetic effects of contaminant exposure, I sequenced mtDNA for 58 BCNH from one reference (Chincoteague Wildlife Refuge,

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This thesis follows the style and format of *Ecotoxicolgy*.

Virginia) and one contaminate (Calumet Lake, Illinois) site and subjected the results to statistical analyses. The statistical tests detect departures from a neutral model and help to explain the differences that might be observed between the two populations (Fu 1997; Okello et al. 2005). Genetic hitchhiking, bottleneck effects, population growth, and background selection can cause departures from neutrality of mutations. Fu and Li's D* and F* tests test against background selection; while TAJIMA's D and $F_S$ test for population growth (Fu 1997).

The first objective of this study was to investigate genetic diversity in a more variable region of the mtDNA than was used by Dahl et al. (2001), who sequenced a 215 base pair region within the Cyt *b* gene. The region of interest, subunit 6 of NADH Dehydrogenase (ND-6), is about 600 base pairs in length. The second objective was to use this region as a biomarker to determine if contaminant exposure affected population genetic patterns.

## MATERIALS AND METHODS

In this study, I used BCNH populations from Chincoteague Wildlife Refuge, Virginia (N = 31) and Calumet Lake, Illinois (N = 27) (Figure 1) for comparison (see Table 1 for amino acid differences, and Appendix I for complete list of samples). Blood and tissue samples from 10-day-old chicks (Dahl et al. 2001), which had been stored at -80ºC, were used for analyses. Custer et al. (1994) explained the collection procedures.

DNA extraction was done using a standard PCI protocol and then quantified by gel electrophoresis on a 0.8% agarose gel and visualized using Eagle Eye II. I then amplified the extracted genomic DNA by using 5µL *Nycticorax* ND6R and 5µL *Nycticorax* Cytochrome b DNF primers in a Polymerase Chain Reaction (PCR) method. The extracted genomic tissue and blood samples each required 5µL dNTP, 5µL buffer, and 0.3µL *Taq* polymerase. One microliter of extracted tissue DNA was used, along with 30µL distilled water; while various amounts of extracted blood DNA were used, along with an adjusted water amount (for exact amounts, see Appendix II), and Mg2/BSA. PCR conditions were as follows: 35 cycles of 95ºC for 15 seconds, 50ºC for 10 seconds, and 72ºC for 1 minute using a GeneAmp PCR System 2700 (Tarr 1995). The resulting product was quantified using gel electrophoresis on a 0.8% agarose gel and visualized using Eagle Eye II. After amplification using 3µL of *Nyc.* Cyt b DNF; PCR products were cut from the gel and cleaned using the QIAquick PCR Purification Kit Protocol. Blood samples, however, were cleaned using the same purification protocol but were not band cut. Amplification of blood samples utilized 3µL of the primer *Nyc.* Proline tRNA Forward. Each sequence was aligned visually, compared and assigned a unique haplotype if differences were observed (Dahl et al. 2001). All unique sequences were

deposited in GenBank. A minimum spanning network showing the relative frequencies of haplotypes in the entire dataset, as well as the evolutionary relationships of haplotypes, was constructed using PAUP* v4.0b10 (see Figure 2) (Swofford, 2002).


**Figure 1.** Map illustrating the two study sites for sampling *Nycticorax nycticorax* populations.

**Table 1.** Variable positions, substitutions and resulting amino acid shifts for the ND6 gene in Black-crowned Night Herons. The variable positions are relative to the crow ND6 sequence in GenBank

| Number | Position | Substitution | Codon Position | Amino Acid |
|---|---|---|---|---|
| 1 | 114 | A → G | 3rd | Val |
| 2 | 144 | T→ C | 3rd | Ala |
| 3 | 187 | T→ C | 1st | Leu |
| 4 | 200 | C→ T | 2nd | Ala → Val |
| 5 | 201 | T→ C | 3rd | Ala |
| 6 | 216 | C→ T | 3rd | Ser |
| 7 | 220 | C→ T | 1st | Pro → Ser |
| 8 | 275 | C→ T | 2nd | Ser → Leu |
| 9 | 307 | C→ T | 1st | Leu → Phe (heteroplasmic) |
| 10 | 138 | A → G | 3rd | Pro |
| 11 | 36 | T→ C | 3rd | Ser |

I analyzed sequence data for both populations with DnaSP 3.5 and ARLEQUINN ver. 2.000 programs. Calculations of haplotype (*h*), Fu and Li's D*, Fu and Li's F*, TAJIMA's D, and $F_S$ values were used to detect genetic differentiation using the coalescent simulations produced by DnaSP 3.5 (Hahn et al. 2002). I used Analysis of Molecular Variance (AMOVA) produced by ARLEQUINN, to test for significant population subdivision.

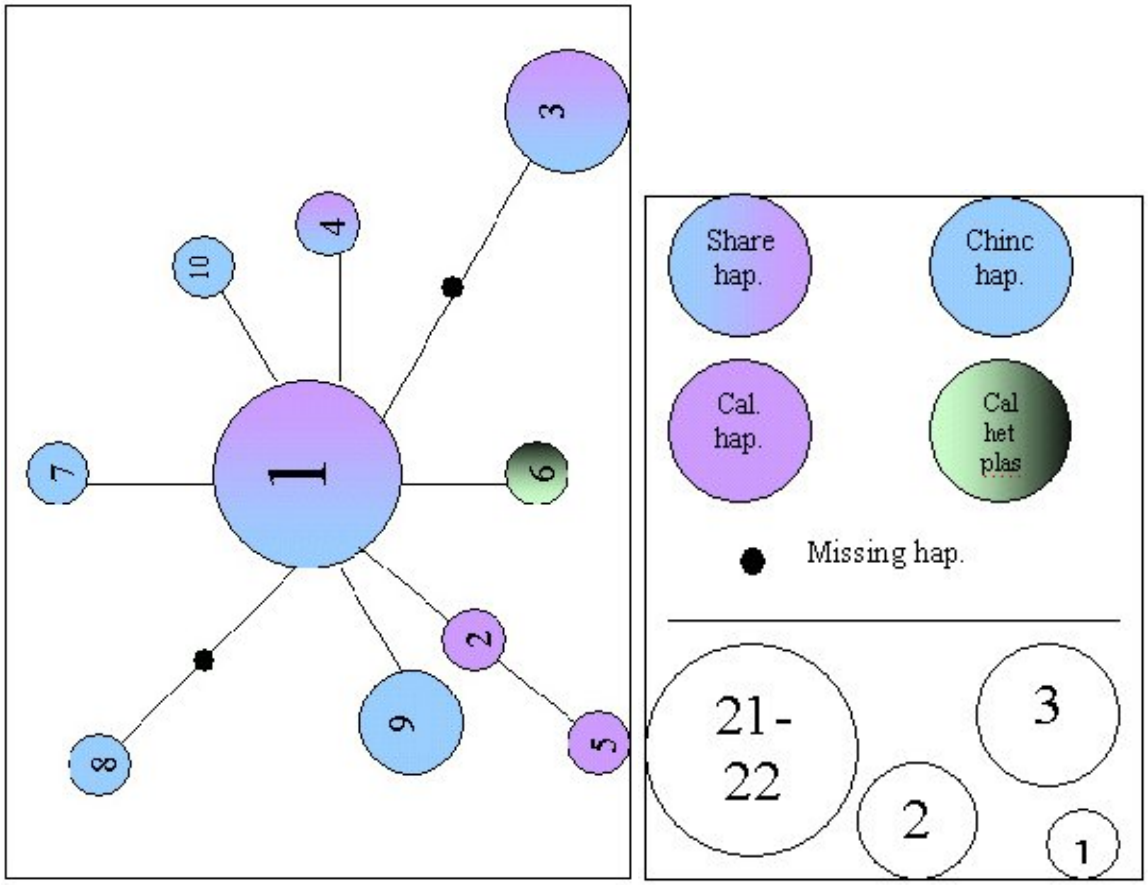


**Figure 2.** A minimum spanning network of relative haplotype frequencies of all BCNH sampled.

## RESULTS

I obtained the nucleotide sequence from a 467 bp segment including 32 bases of the $tRNA^{Pro}$, 11 bases of an intergenic spacer, and 423 bases of the 3' end of the ND-6 gene from each of the 58 specimens studied. Eleven variable positions defined 10 haplotypes (Table 1). All of the variable positions were in the ND6 gene. The 11 changes included nucleotide substitutions at three $1^{st}$ codon positions, two at $2^{nd}$ codon positions, and six at $3^{rd}$ codon positions, and they coded for four amino acid replacements. The two populations shared three of the 10 haplotypes (Table 2). This included a single common haplotype (haplotype 1); the other nine haplotypes were present in low frequencies (N = 1-3 per population). Chincoteague had four private haplotypes, and Calumet had three. A single haplotype (haplotype 6) from Calumet was observed in one heteroplasmic individual.

Table 3 presents the results of one coalescent simulation from combining the two sites to act as one population using the DnaSP program. Coalescence results estimate the expected number of haplotypes to be seven, yet 10 haplotypes were observed. This indicates both populations have experienced expansion and there is an excess of unique haplotypes. Coalescent Fu and Li's F* and TAJIMA's D values were both significant, also indicating population expansion. Fu and Li's D* was not significant at the 95% confidence level, but it was significant at the 90% level. Such a borderline significant result could indicate that sample size was not sufficient for this test.

The value of $F_S$, which tests for genetic hitchhiking and population growth, was significant and negative, implying population growth (Fu 1997) and not genetic hitchhiking or background selection (Okello et al. 2005). A significant and positive

result in this test would have indicated the population was evolving in a non-neutral pattern and acted upon by background selection (Fu 1997).

Analysis of molecular variance across all loci did not show significance (Table 4); yet the locus-by-locus AMOVA was significant (Table 5). This indicates an absence of population subdivision.

Although, AMOVA indicated that there was no population subdivision, coalescent simulations for each site were also run. Results of these simulations did not show significance for any neutrality test (Table 6), unlike the combined data simulations. Reasons for this difference could be due to insufficient sample size.

**Table 2.** Haplotype relative frequency distribution among the samples

| Haplotype | Chincoteague (n=31) | Calumet (n=27) |
|---|---|---|
| Hap 1 | 0.71 | 0.778 |
| Hap 2 | 0 | 0.037 |
| Hap 3 | 0.0968 | 0.0741 |
| Hap 4 | 0.0323 | 0.037 |
| Hap 5 | 0 | 0.037 |
| Hap 6 | 0 | 0.037 |
| Hap 7 | 0.0323 | 0 |
| Hap 8 | 0.0323 | 0 |
| Hap 9 | 0.0645 | 0 |
| Hap 10 | 0.0323 | 0 |

**Table 3.** Results of the coalescent simulations based upon 1000 replicates, no recombination, and value of Theta=0.7308

| | Fu and Li's D* | Fu and Li's F* | $F_S$ | TAJIMA's D | h | Hd |
|---|---|---|---|---|---|---|
| Obs. Value | -2.1300 | -2.4517 | -6.9764 | -1.9652 | 10 | 0.4465 |
| CI Lower | 0.02348 | -2.38164 | -3.62380 | -1.47828 | 1 | 0 |
| CI Higher | 0.75525 | 1.56806 | 4.76400 | 2.02191 | 7 | 0.76165 |

**Table 4.** Analysis of molecular variance as a combined population across 11 loci

| Source of Variation | d.f. | Sum of Squares | Variance Components |
|---|---|---|---|
| Among populations | 1 | 0.357 | -0.00353 |
| Within populations | 56 | 25.691 | 0.45877 |
| Total | 57 | 26.048 | 0.45524 |
| Fixation Index $F_{ST}$: | -0.00776 | | |

**Table 5.** Locus-by-locus analysis of molecular variance across 11 loci

| Source of Variation | d.f. | Sum of Squares | Variance Components |
|---|---|---|---|
| Among populations | 1 | 0.254 | -0.00392 |
| Within populations | 56 | 20.573 | 0.36738 |
| Total | 57 | 20.828 | 0.36346 |
| Fixation Index $F_{ST}$: | -0.01080 | | |

**Table 6.** Results of the coalescent simulations for each site based upon 1000 replicates and no recombination

| Site | Fu and Li's D* | Fu and Li's F* | $F_S$ | TAJIMA's D |
|---|---|---|---|---|
| Chincoteague | -1.87296 | -2.16101 | -3.483 | -1.79750 |
| Calumet | -1.04646 | -1.44285 | -3.175 | -1.70783 |

## DISCUSSION AND CONCLUSIONS

Wildlife populations exposed to genotoxic contaminants have an increased risk of induced mutations that can increase variability, as well as bottleneck effects that can result in the reduction of genetic variability (Dahl et al. 2001). Whereas increased mutation rates could lead to potential catastrophic population crashes due to mutational meltdown (Lynch et al. 1995), a bottleneck can lead to the reduction of genetic diversity, and decrease a population's overall fitness from increased levels of inbreeding (Saillant et al. 2004).

In comparison to Dahl et al. (2001), this study revealed relatively high levels of genetic variation in BCNH. However, results indicated no significant genetic differentiation between the two populations. This could be the result of demographics, such as their ability to disperse, or to historical factors. The Chincoteague population and the Calumet population are over 1500 miles distant and they are located in separate migratory flyways. They occupy very distinct environments: Chincoteague has remained pristine and relatively unaffected by industrial activities; while Calumet Lake has been impacted by industrial waste, heavy metals, and urbanization. It is not known to what extent BCNH populations might adapt to local environmental conditions. Gene flow could alter the genetic diversity of local populations and prevent significant genetic differentiation. If local populations receive numerous immigrants, it could have resounding effects on gene diversity in either a positive or negative way, depending on the source populations (Matson et al. 2000). Due to the limited population sampling in this study, it is impossible to say if the lack of subdivision and high diversity levels indicate any effects of contaminant exposure. The observation of a single heteroplasmic

individual and slightly higher number of haplotypes at Calumet suggest such effects, but this will require examination of larger sample sizes and more populations to confirm.

This study examined 10-day-old chicks whose exposure levels should reflect that of their local environment (Custer et al. 1994). No convincing evidence of an increased mutation rate was observed in this study or in that of Dahl et al. (2001), notwithstanding the fact that the Calumet population was chosen for analysis based upon considerable evidence of biomarker effects and contaminant exposure. The relationship between exposure, somatic effects, and induced heritable mutations is difficult to establish. Nonetheless, several recent studies convincingly show induced mutations in both natural wildlife populations (Yauk and Quinn 1996; Yauk et al. 2000; Matson et al. 2004) and laboratory mice exposed *in situ* to contaminants (Somers et al. 2002).

The coalescent simulation results indicate that a significantly increased number of haplotypes could be due to population growth. Fu and Li's F* and TAJIMA's D were marginally significant which also supports a scenario of population expansion. And, finally, Fs were significant and negative indicating population growth. These analyses present a consistent picture of the demography, which will be useful in future genetic studies of this important sentinel species. This historical population expansion has resulted in a pattern with a single predominant haplotype (haplotype 1), and a large number of low frequency haplotypes. Since bottlenecks can effectively eliminate rare alleles, BCNH populations should be sensitive indicators of avian population declines resulting from contaminant exposure or other anthropogenic changes in the environment.

The main objective of this study was to develop a biological marker in mitochondrial DNA for use as an indicator for contamination assessment. The results,

which show high diversity for the ND6 gene of the mtDNA, succeeded in this endeavor. Although, mtDNA is hypothesized to reveal increased mutations when exposed to genotoxic contaminants, this is not always the case, as was seen in this study and that of Johnson et al. (1999). The results of her research showed similar results of no significant genetic diversity among Wood Duck (*Aix spopnsa*) populations. Nonetheless, levels of diversity for ND6 are high enough in BCNH to serve as a useful biomarker of population effects.

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# APPENDIX I

Black-crowned Night Heron Sample Collection

| Sample # | Specimen # | Locality | Haplotype | Collection Year |
|---|---|---|---|---|
| 1 | IL02-B | Calumet Lake | 1 | 2002 |
| 3 | IL05-B | Calumet Lake | 1 | 2002 |
| 4 | IL06-B | Calumet Lake | 1 | 2002 |
| 5 | IL07-B | Calumet Lake | 1 | 2002 |
| 6 | IL08-B | Calumet Lake | 6 | 2002 |
| 7 | IL09-B | Calumet Lake | 1 | 2002 |
| 8 | IL11-B | Calumet Lake | 1 | 2002 |
| 9 | IL12-B | Calumet Lake | 1 | 2002 |
| 10 | IL13-B | Calumet Lake | 1 | 2002 |
| 11 | IL14-B | Calumet Lake | 3 | 2002 |
| 12 | IL15-B | Calumet Lake | 1 | 2002 |
| 13 | IL16-B | Calumet Lake | 3 | 2002 |
| 14 | IL17-B | Calumet Lake | 1 | 2002 |
| 16 | VA62-B | Chincoteague | 1 | 2002 |
| 17 | VA63-B | Chincoteague | 1 | 2002 |
| 18 | VA64-B | Chincoteague | 1 | 2002 |
| 19 | VA65-B | Chincoteague | 1 | 2002 |
| 20 | VA66-B | Chincoteague | 4 | 2002 |
| 21 | VA67-B | Chincoteague | 1 | 2002 |
| 22 | VA68-B | Chincoteague | 1 | 2002 |
| 23 | VA69-B | Chincoteague | 1 | 2002 |
| 24 | VA70-B | Chincoteague | 1 | 2002 |
| 25 | VA72-B | Chincoteague | 1 | 2002 |
| 26 | VA73-B | Chincoteague | 3 | 2002 |
| 27 | VA74-B | Chincoteague | 1 | 2002 |
| 28 | VA75-B | Chincoteague | 1 | 2002 |
| 29 | VA77-B | Chincoteague | 10 | 2002 |
| 30 | VA79-B | Chincoteague | 8 | 2002 |
| 31 | VA80-B | Chincoteague | 1 | 2002 |
| 32 | 91CBP1 | Chincoteague | 1 | 1991 |
| 33 | 91CBP5 | Chincoteague | 1 | 1991 |
| 34 | 91CBP6 | Chincoteague | 1 | 1991 |
| 35 | 91CBP8 | Chincoteague | 1 | 1991 |
| 83 | 91CBP10 | Chincoteague | 1 | 1991 |
| 85 | 91CBP13 | Chincoteague | 1 | 1991 |
| 86 | 91CBP15 | Chincoteague | 1 | 1991 |
| 36 | 91CBP17 | Chincoteague | 9 | 1991 |
| 37 | 91CBP20 | Chincoteague | 1 | 1991 |
| 38 | 91CBP21 | Chincoteague | 7 | 1991 |
| 39 | 91CBP25 | Chincoteague | 1 | 1991 |
| 40 | 91CBP26 | Chincoteague | 1 | 1991 |
| 82 | 91CBP27 | Chincoteague | 1 | 1991 |
| 41 | 91CBP28 | Chincoteague | 9 | 1991 |

| Sample # | Specimen # | Locality | Haplotype | Collection Year |
|---|---|---|---|---|
| 42 | 91CBP31 | Chincoteague | 1 | 1991 |
| 44 | 91CBP33 | Chincoteague | 1 | 1991 |
| 45 | 91CBP36 | Chincoteague | 3 | 1991 |
| 46 | 91CBP42 | Chincoteague | 1 | 1991 |
| 47 | 91CBP43 | Chincoteague | 3 | 1991 |
| 49 | BC03EBC | Calumet Lake | 1 | 1993 |
| 52 | BC06EBC | Calumet Lake | 4 | 1993 |
| 53 | BC07EBC | Calumet Lake | 1 | 1993 |
| 54 | BC08EBC | Calumet Lake | 1 | 1993 |
| 55 | BC09EBC | Calumet Lake | 1 | 1993 |
| 56 | BC10EBC | Calumet Lake | 1 | 1993 |
| 60 | BC15EBC | Calumet Lake | 5 | 1993 |
| 61 | BC17EBC | Calumet Lake | 1 | 1993 |
| 62 | BC18EBC | Calumet Lake | 2 | 1993 |
| 63 | BC20EBC | Calumet Lake | 1 | 1993 |

## APPENDIX II

Specific Blood Sample Amounts and Distilled Water Used During PCR

| Sample # | µL DNA | µL Water |
|---|---|---|
| 1 | 12 | 0 |
| 2 | 12 | 0 |
| 3 | 12 | 0 |
| 4 | 12 | 0 |
| 6 | 12 | 0 |
| 8 | 12 | 0 |
| 9 | 8 | 4 |
| 10 | 12 | 0 |
| 11 | 12 | 0 |
| 12 | 12 | 0 |
| 13 | 6 | 6 |
| 14 | 6 | 6 |
| 15 | 8 | 4 |
| 16 | 8 | 4 |
| 17 | 8 | 4 |
| 18 | 12 | 0 |
| 19 | 12 | 0 |
| 20 | 8 | 4 |
| 21 | 12 | 0 |
| 22 | 8 | 4 |
| 23 | 8 | 4 |
| 24 | 12 | 0 |

| Sample # | µL DNA | µL Water |
|---|---|---|
| 25 | 12 | 0 |
| 26 | 4 | 8 |
| 27 | 8 | 4 |
| 28 | 12 | 0 |
| 29 | 8 | 4 |
| 30 | 12 | 0 |
| 31 | 8 | 4 |
| 35 | 4 | 8 |
| 47 | 3 | 9 |
| 48 | 12 | 0 |
| 50 | 12 | 0 |
| 52 | 12 | 0 |
| 53 | 4 | 8 |
| 54 | 6 | 6 |
| 55 | 4 | 8 |
| 56 | 3 | 9 |
| 57 | 12 | 0 |
| 58 | 12 | 0 |
| 59 | 12 | 0 |
| 60 | 6 | 6 |
| 61 | 12 | 0 |
| 62 | 12 | 0 |
| 63 | 3 | 9 |

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