SOUTHWEST FISHERIES CENTER

NATIONAL MARINE FISHERIES SERVICE SOUTHWEST FISHERIES CENTER P.O. BOX 271 LA JOLLA, CA 92038

JANUARY 1981

PRELIMINARY ANALYSIS OF THE POTENTIAL FOR STOCK ASSESSMENT OF PACIFIC OCEAN DELPHINIDS BY G AND C CHROMOSOME BANDING

BY

GARY L. WORTHEN

ADMINISTRATIVE REPORT NO. LJ-81-02C

Final Report on

National Marine Fisheries Service

Contract No. NOAA/79-ABC-00205

PRELIMINARY ANALYSIS OF THE POTENTIAL FOR STOCK ASSESSMENT OF PACIFIC OCEAN DELPHINIDS BY G AND C CHROMOSOME BANDING

By

Gary L. Worthen, Research Associate
Biomedical Laboratory, Exceptional Child Center
Utah State University
Logan, Utah 84322

This contract was let to assess the potential of chromosomal banding analyses in defining subspecific units of pelagic dolphins in the eastern tropical Pacific. To conduct the necessary research, the investigation collected tissue samples from accidentally killed dolphins and conducted storage viability tests and cytogenetic analyses on them. Recommendations are made for a follow-up study.

TABLE OF CONTENTS

List of Tables

List of Figures

Abstract

Despite considerable effort expended in assessing and defining stocks of dolphins in the eastern tropical Pacific, the current most pressing biological research problem is definition of stocks. To help in this effort, I accompanied a working tuna seiner (the M/V Maria C. J.) from October 7 to November 2, 1979. Protocols were established and utilized for the removal of sterile tissue samples from sixteen accidentally killed dolphins: seven northern offshore spotted dolphins (Stenella attenuata) and nine eastern spinner dolphin (Stenella longirostris). A total of 248 tissue samples of various kinds were extracted from the 16 animals using standard sterile techniques. These samples (consisting of skin, lung, kidney, heart, cornea, and embryo) were stored under one of several test regimens: cryogenic freezing, refrigeration, and ambient air conditioned room temperature. Under the latter two temperature regimens, both McCoys and L-15 media were used. Tissue viability under each of these storage regimens was analyzed. Lung and embryonic tissue were the only tissue types which survived the storage and shipping to produce viable tissue cultures. Storage in McCoys medium under refrigeration was determined to be better than other storage protocols. An attempt was made to analyze blood leucocytes on board the ship but this attempt met with failure, most probably as a result of fluctuations in the temperature of the incubator which resulted from power fluctuations on board the ship.

Successful tissue cultures were obtained from five of the accidentally killed dolphins: four Stenella attenuata and one Stenella longirostris. A third species (S. plagiodon) from the Atlantic was used as a base for comparison. Standard G- and C-band analyses were conducted in addition to an assessment of the NORs for these six animals.

All three species show a 2N number of 44 chromosomes, corresponding to the diploid number found in most cetaceans. The autosome complement contains 6 metacentric, 8 sub-metacentric, 2 sub-telocentric, and 5 telocentric pairs. The X is the largest metacentric chromosome in the spread and (in males) the minute Y is by far the smallest chromosome. Two chromosomal aberrations were noted: a balanced translocation was observed in the S. longirostris and an inversion in one of the S. attenuata. Both aberrations were found in all cells examined and were apparently present in the animals rather than being caused by the vicissitudes of cell culture. None of the three species showed significant variability in either G-band or NOR analyses. Considerable interspecific variability was observed in the amount and placement of constitutive heterochromatin (C-bands). Intraspecific variability in the amount of heterochromatin present was also observed in S. attenuata. Considerable heteromorphism was observed in the amount of heterochromatin of homologous chromosomes in the same animal. This heteromorphism in C-banded material was reflected in a corresponding heteromorphism in the amount of G-negative material on the homologous chromosomes. The significance of the observed constitutive heterochromatin variation is discussed in light of salient literature and its predicted ability as a tool for future research in defining impacted stocks of dolphins in the eastern tropical Pacific. Additionally, an experimental design and field sampling protocol is presented for a proposed follow-up study.

1.0 Introduction

1.1 Background

Dolphins have long been a source of curiosity and an object of study by scientists. Early works on cetacean systematics and phylogeny date back to the early classics of Flower (1883) and True (1889). Additional works which serve as milestones in cetacean taxonomy are those of Winge (1921), Kellogg (1928), Slijper (1936), Simpson (1945), Tomilin (1957), Fraser and Purves (1960), Nishiwaki (1963), Fraser (1966), Rice and Scheffer (1968), Mead (1972), Nishiwaki (1972), Kasuya (1973), Schenkkan (1973), Yablokov, et al., (1974), Mitchell (1975), and Rice (1976). Of those papers listed above, several deal specifically with the family Delphinidae: Flower (1883), True (1889), Nishiwaki (1963) and Fraser (1966).

In order to elucidate the relationships between various taxa within the Cetacea, many workers have undertaken cytogenetic studies. These works include those of Makino (1948), Atwood and Rozavi (1965), Walen and Madin (1965), Carr, et al. (1966), Kasuya (1966), Duffield, et al. (1967), Horral, et al. (1968), Arnason (1969), (1970), Prasad, et al. (1970), Duffield Kulu, et al. (1971), Duffield Kulu (1972), Arnason (1972), Arnason and Benirschke (1973), Arnason (1974), Duffield (1974), (1977), Arnason, et al. (1977), Arnason, et al. (1978), and Arnason (1980). Many of the older works listed above used only gross karyotyping as a means of comparison and as such are subject to reinvestigation.

The system of classification used in this work follows that outlined by Walker, et al. (1975) in which Stenella attenuata and Stenella plagiodon are considered distinct species, instead of the nomenclature given by Rice (1976)

in which S. attenuata and S. plagiodon are both lumped under the rubric of S. dubia. In either system S. longirostris is considered a distinct species.

The effort leading to the inception of this study began with the proposal "Stock Assessment of Pacific Ocean Delphinids by G and C Chromosome Banding", written in response to National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Solicitation No. NASO-79-00205. The proposal survived preliminary screening; but, as a result of financial constraints in the National Marine Fisheries Service, was revised and resubmitted with an amended budget. The amended proposal led to the contract award.

1.2 Conceptual Basis for the Study

Despite considerable effort expended in assessing and defining stocks of dolphins in the eastern tropical Pacific, much is yet to be determined on virtually every species. The existing level of knowledge is well defined in Smith (1979), and a representative example of the distribution charts that this work contains for the most common species of delphinids is reproduced here (see Figures 1 and 2).

Perrin (1977:52) in discussing research on dolphins indicates that one of the most pressing current biological research problems is definition of stocks. The two major problems he mentions in stock definition are the nature of the boundaries between the eastern and white belly spinner dolphin (Stenella longirostris) (see Figure 2), and the possible existence of a relatively unfished and morphologically distinct southwestern form of the spotted dolphin (Stenella attenuata) (see Figure 1). Evidence of possible genetic distinction between stocks may be found in the large areas of sympatry between them. Such wide areas of overlap (see Figure 2) either indicate a

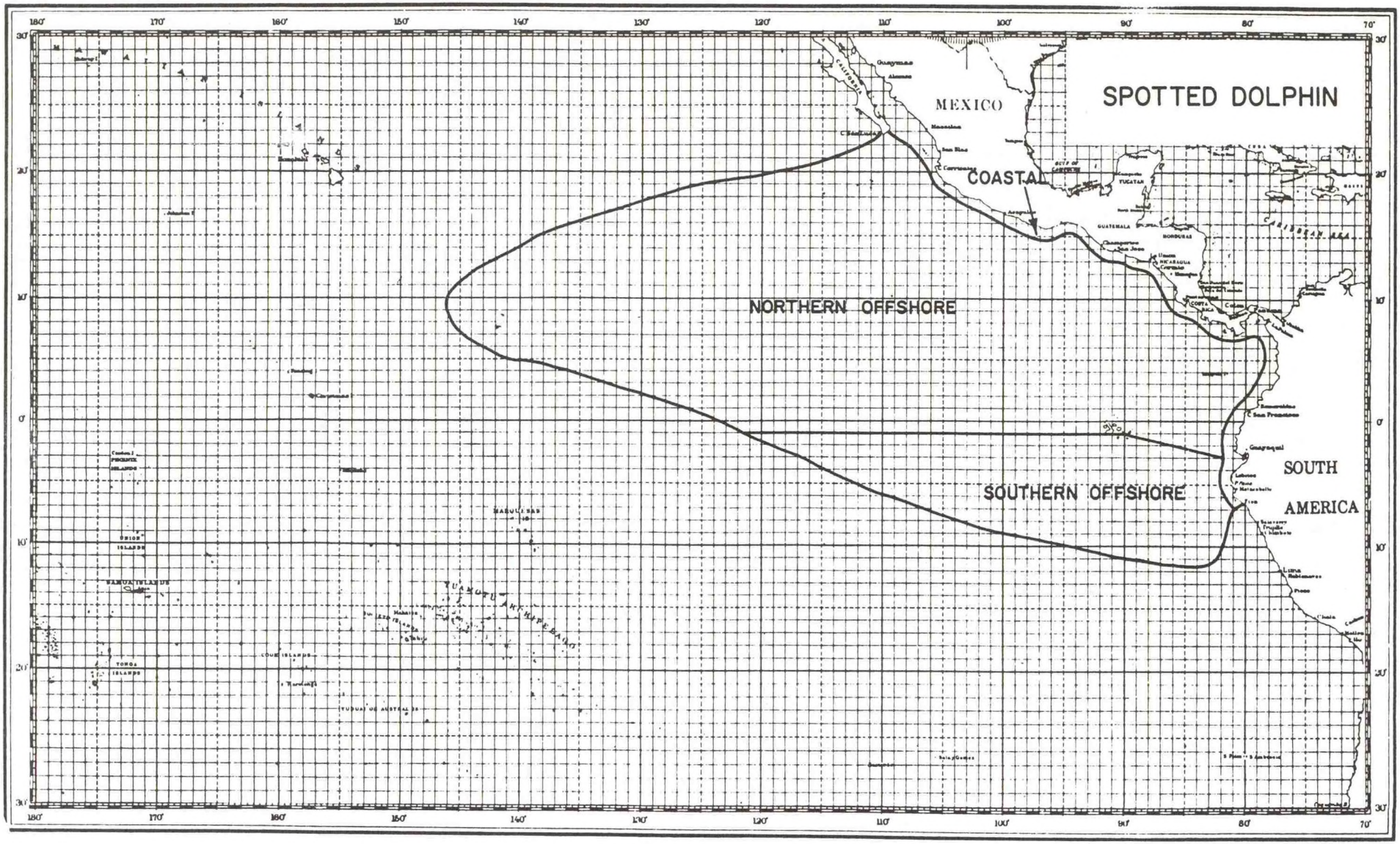


Figure 1. Distribution of the several stocks of spotted dolphin, Stenella attenuata, in the eastern Pacific Ocean (after Smith, 1979).

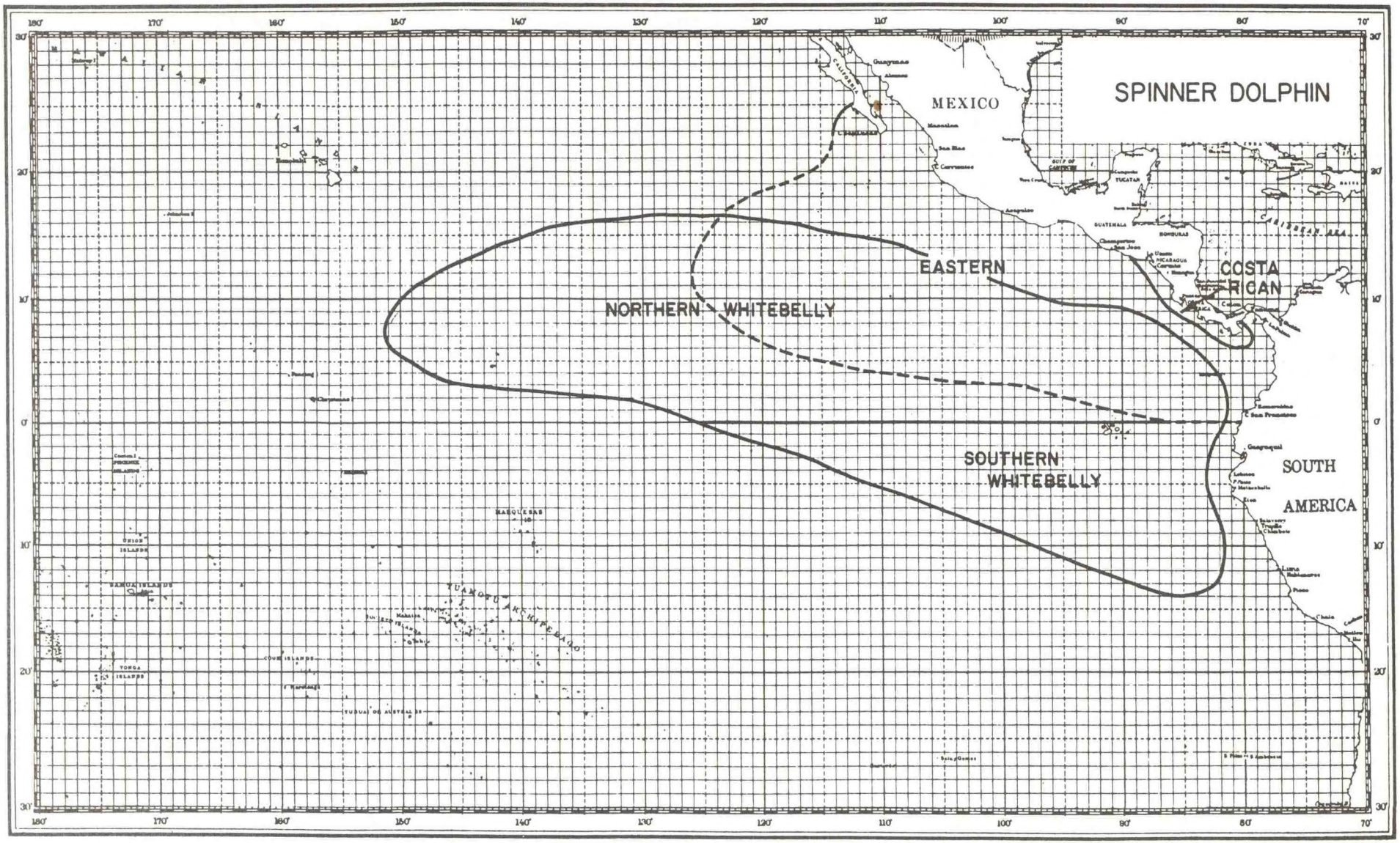


Figure 2. Distribution of the several stocks of spinner dolphin, Stenella longirostris, in the eastern Pacific Ocean (after Smith, 1979).

broad zone of intergradation with no restriction in gene flow or two rather (or totally) genetically isolated populations which are partially sympatric.

Karyotypic analysis is a potential tool for evaluating the validity of present stock definitions and boundaries for dolphins involved in the purse seine fishery for tuna. Existing boundaries have been drawn based on external and skeletal morphology and on distributional patterns. An additional and independent line of evidence, such as chromosome morphology, may suggest that some boundaries should be eliminated or that some new ones should be erected. Karyotypic analysis may also allow assessment of the relationships among the stocks, and afford insight into their origins and dynamic zoogeography.

1.3 Objectives

The major stated objectives of this research were intended to encompass two years of investigation. As this is the final report for the successful completion of the first year of the proposed two-year study, only those objectives relevant to the first year are discussed here.

The major overall objective of this study was to assess the potential of chromosomal banding analysis in defining subspecific units of pelagic dolphins in the eastern tropical Pacific. In order to adequately meet the research objective, the following task objectives are indicated.

1. We were to compile an annotated bibliography on the use of karyotypic analysis in the subspecific taxonomy of mammals. To complete this task we conducted a computerized bibliographic search of titles and abstracts, references found in citation in known relevant articles, and manual searches. All available articles found in such a manner were obtained and a brief abstract written (or taken from the article itself) for use as an annotation. The final product consists of a report of 118 pages (and 36

pages of appendices) which summarizes 127 references. These references are all contained in each of five separate listings: by author (including all junior authors), date, key word within the context of the remainder of the title of each article, subject, and a full alphabetical annotated bibliographic listing.

2. We were to accompany a NMFS-chartered cruise to the eastern tropical Pacific to assess the field sampling situation on board a working tuna purse-seiner and to collect initial tissue samples. Both facets of this objective have been successfully completed (see Sections 2.0 and 3.0, below).

3. We were to analyze initial samples for quality and for the potential use of chromosomal banding in identifying stocks of dolphins in the eastern tropical Pacific. This objective has been met (see Sections 4.0, 5.0, 6.0, and 7.0, below).

4. We were to prepare a report on the potential of the method, and, if the potential was judged to be large, to design an experiment and a field sampling protocol to define stocks using samples to be collected in the NMFS observer program. This writing constitutes that report and Section 8.0, below, presents recommendations for future research.

1.4 Acknowledgements

This work was supported under National Oceanic and Atmospheric Administration Contract No. 79-ABC-00205. I would like to express appreciation to all those at the National Marine Fisheries Service, Southwest Fisheries Center who have aided in any way in this research. I would particularly like to express gratitude to Dr. William F. Perrin for his continued interest and support throughout the investigation. I would also

like to thank James Coe and Larry Hansen for their assistance in preparing me for the cruise on the M/V Maria C. J. I wish to express gratitude to the captain and crew of the M/V Maria C. J. for their help, cooperation, and courtesy while I was on board the seining vessel. In particular, I wish to express gratitude to Captain Jose Jorge for his amiable cooperation with the research project, and to the Chief Engineer, Elias Silveira for allowing me to set up a small research station in his private room. I wish to express special thanks to Mr. Darrel Lee of the National Marine Fisheries Service for his help and cooperation in gathering tissue samples and his advice and camaraderie while I was on board the tuna vessel.

I wish to express gratitude to members of our research staff and support staff at Utah State University for their help and cooperation in the completion of this work. I wish to express special thanks to Dr. Wilbur S. Thain for his constant encouragement, support and input throughout the entire project. I would like to thank the following individuals for their careful reading of the manuscript: Dr. Wilbur S. Thain, Utah State University Biomedical Laboratory; Scott R. Woodward, Utah State University Biology Department; Dr. John B. Mailhes, Louisiana State University Medical Center; Dr. Kurt Benirschke, Zoological Society of San Diego; Dr. William F. Perrin, Dr. Tim Smith, Dr. Al Myrick, and Dr. Warren Stuntz, National Marine Fisheries Service Southwest Fisheries Center. I also wish to express gratitude to members of our support staff without whose help timely completion of the project would have been impossible. In particular, I would like to thank Marilyn Tinnakul, Connee Polak, Phyllis Herr, Ingrid Hansen, Kelli Cordon, and Ginny Tolfa.

2.0 Assessment of Field Sampling Environment

2.1 Tuna Fishing Vessels

I spent from October 7, 1979 to November 2, 1979 on board a working tuna vessel (the M/V Maria C. J.). My participation in the cruise was essential in order for us to assess the field sampling environment found on a working tuna vessel. I found tuna fishing vessels to present, at best, an unsterile environment; and the only possibility of achieving any degree of sterility on board was to make a small area on the working deck (where the tissue samples were gathered) a reasonably sterile environment. The captain and crew of the M/V Maria C. J. and the NMFS observer on board were very cooperative in their efforts to assist me in the gathering of the necesary tissue samples. Using the M/V Maria C. J. and the M/V Elizabeth C. J. as my only base for comparison, the following observations can be made.

It is virtually impossible to remove tissue samples from accidentally killed dolphins until such time as the fishing operation on a particular set has been completed. During the set, while the tuna (and therefore the dolphins--if present) are being brailed aboard, the presence of an observer or scientist removing tissue samples on the work deck would not be tolerated by most captains and crews. Secondly, the conditions on the work deck while the actual fishing operation is being undertaken decreases the possibility of achieving a reasonably sterile environment on the work deck. The level of activity by the fishermen would in itself preclude tissue removal at that time but such factors as water misting from the nets over virtually all of the work deck would also be a very real problem in contamination.

I found the ideal time to remove the tissue samples is immediately after the set has been entirely completed and the decks have been washed down and

cleaned as is done after each set. Attempts can then be made to make a small sterile environment on board the work deck in the immediate vicinity of where the tissue samples are to be removed. Although total sterility on board the work deck is impossible, I found that the area can be cleansed to the degree that the majority of tissue samples can be collected in a sterile or reasonably sterile condition. The lack of sterility on the tuna vessels, therefore, does not seem to pose an insurmountable obstacle.

2.2 NMFS Observers

At the inception of the study, our single largest concern was the on-board, tissue-gathering phase of the research. We felt that the success or failure of the entire project rested with the techniques, procedures, skill, and dedication employed by the person on-board gathering the samples. We did not know what conditions exist on tuna seiners, as we had no experience in this area and first hand observation and interactive decision making were considered great benefits to the project.

As a result of my participation on the research cruise, we now have concrete ideas of the working environment on board ship and therefore the most feasible techniques to be used in the removal and storage of tissue samples. I, furthermore, had the opportunity to interact with one of the NMFS observers, Darrel Lee. I worked with Mr. Lee enough to be able to assess the level of cooperation that could be expected from at least some of the observers. From these experiences and interactions as well as testing our trial techniques for tissue sample removal, it would appear that there should be no problem in training NMFS observers to successfully remove tissue samples from accidentally killed dolphins.

3.0 Tissue Samples Obtained

3.1 Structure of the Kill

A total of 16 dolphins, consisting of one population from each of two species, were accidentally killed during the normal course of four sets while I was on board the M/V Maria C. J. (see Table 1 and Figure 3). Nine of the animals were eastern spinner dolphin (Stenella longirostris). All but one of the Stenella longirostris were killed in one set, Set #16. Seven of the animals killed were northern offshore spotted dolphins (Stenella attenuata). Eleven of the animals were sub-adults ranging from neonates to animals of unknown age (speckled juveniles or two tone animals). Five of the animals killed were adults. Two of those five were adult females and one of those adult females was gravid and contained a small and partially developed embryo approximately 30 cm. in length. Copies of the completed life history forms for each of the sixteen accidentally killed dolphins appear in Appendix A.

The temporal distribution of the kill was rather erratic. One dolphin (original collection No. DEL031) was killed on October 12, 1979, early in the trip. No other dolphins were killed until October 17, 1979 when a total of 12 animals were killed in a single set (Set #16). Slightly more than a week later (on October 25, 1979) a total of three animals were killed in two separate sets (Sets #32 and #33). With the number of animals to be processed in Set #16, it was impractical to gather each tissue type from each animal that was killed; especially if additional dolphins were expected to be killed during the trip. The total number of vials which would have resulted from such sampling and which would have needed to have been processed in our laboratory would have been prohibitive. Therefore, a random sampling of tissue was removed from the 12 animals in question. Similar modus operandi

Table 1. Structure of the kill for accidentally killed dolphins taken on a working tuna seiner (the M/V Maria C. J.) in the eastern tropical Pacific Ocean on cruise number 565.*

SET 3		13° 50' N LAT.	106° 08' W LONG.	10/12/79
(D1)	1-2 year old male	Offshore	Stenella attenuata	(DEL031)
SET 16		09° 34' N LAT.	107° 29' W LONG.	10/17/79
(D2)	Juvenile female	Eastern	Stenella longirostris	(DEL032)
(D3)	Juvenile female	"	"	(DEL033)
(D4)	Juvenile male	"	"	(DEL034)
(D5)	Adult female	"	"	(DEL035)
(D6)	Juvenile female	"	"	(DEL036)
(D7)	Juvenile male	"	"	(DEL037)
(D11)	Juvenile female	"	"	(DEL041)
(D12)	Juvenile female	"	"	(DEL042)
(D8)	Neonate female	Offshore	Stenella attenuata	(DEL038)
(D9)	Two tone male	"	"	(DEL039)
(D10)	Juvenile female	"	"	(DEL040)
(D13)	Adult male	"	"	(DEL043)
SET 32		09° 38' N LAT.	110° 00' W LONG.	10/25/79
(D14)	Adult male	Offshore	Stenella attenuata	(DEL044)
(D15)	Adult female	"	"	(DEL045)
(D16)	Embryo male	"	"	(**)
SET 33		09° 52' N LAT.	110° 24' W LONG.	10/25/79
(D17)	Adult male	Eastern	Stenella longirostris	(DEL046)

*For each animal the accession number (D__), age (as supplied by the NMFS observer), sex, population (stock), species, and the NMFS observer's collection number (DEL___) is given.

**No collection number was assigned to this animal as it was an embryo removed from D15 (DEL045).

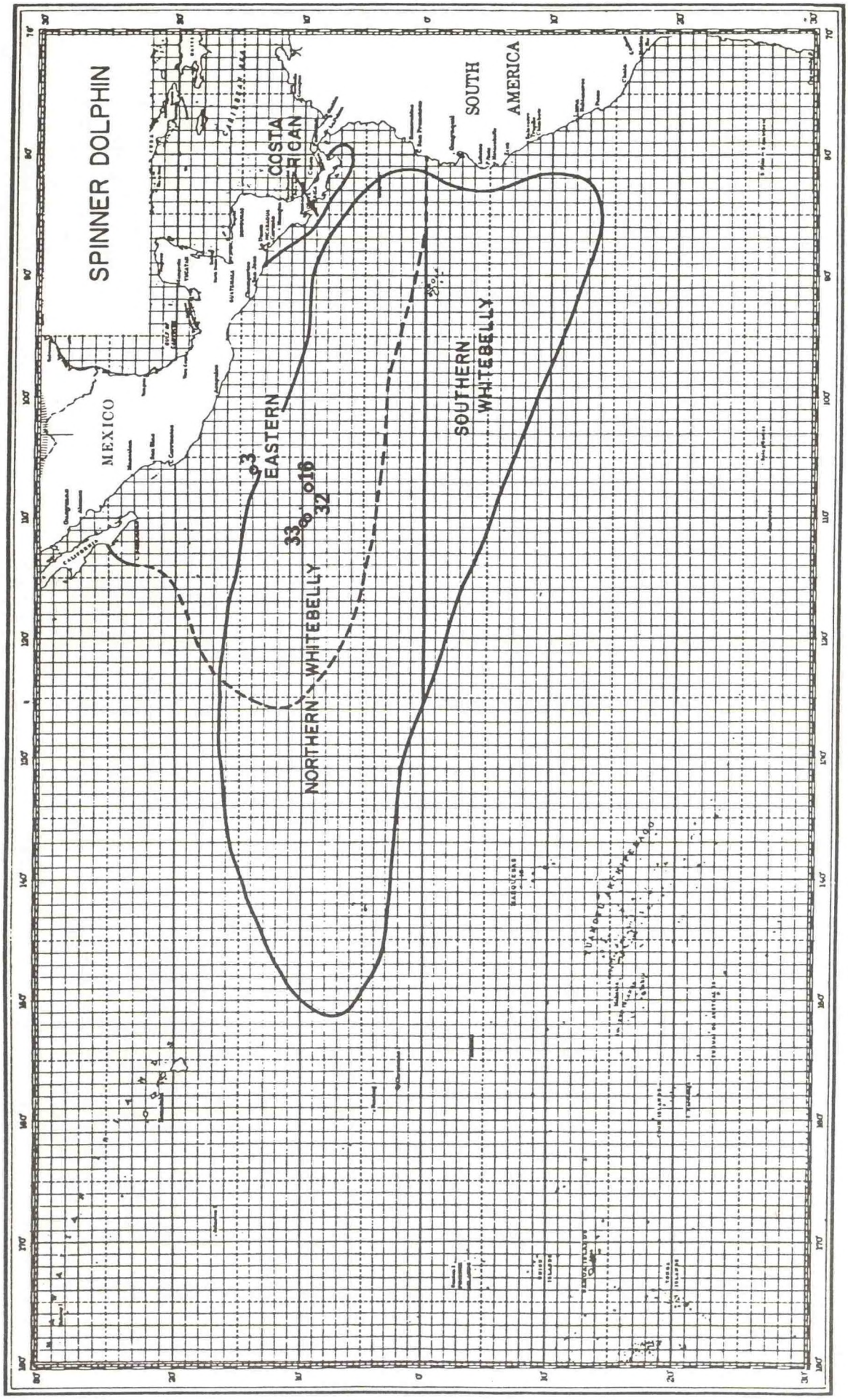


Figure 3. Locations of sets (by set number) in which dolphins were accidentally killed in relationship to the recorded distribution of the spinner dolphin, Stenella longirostris (after Smith, 1979).

were employed on the three animals killed in Sets #32 and #33. Both the observer and myself fully expected that we would obtain more animals and random sampling procedure was deemed to be the best possible approach. However, no additional animals were killed after October 25 on the M/V Maria C. J. Cruise No. 565.

At the inception of the study we were concerned about the possibility of not obtaining an adequate sample of dolphins from appropriate dolphin stocks to completely conduct our research. We desired a sampling of animals from as many different stocks as feasible and hoped that at least two stocks of at least two of the major species would be sampled. We hoped to sample at least three to six animals (more if available) of each stock of animals of interest (S. attenuata and S. longirostris). We felt it was much more important for us to obtain samples from as many stocks as possible from one or two species than to obtain samples from only one stock of a large number of species.

From the information given in Table 1, it is apparent that we did not obtain as large a sample as we would have liked, and that the temporal distribution of the kill (with 12 animals coming in one batch) further hampered our efforts to obtain as many tissue types as possible. These problems can be entirely eliminated in the subsequent studies as we now have very firm ideas of which tissue types to gather, facilitating quick removal of those tissues.

3.2 Materials and Methods

Tissue samples of varying types and numbers were removed from all the accidentally killed dolphins after the NMFS observer, Darrel Lee, obtained the life history data which were needed. Biomedical Laboratory

data accession forms (see Figure 4) were filled out at this time for each animal. Mr. Lee then assisted me in the physical handling of the dolphins, vials, and ethanol (95% ethyl alcohol) to help ensure the sterility of the samples. The general procedures which we used to assure the sterility of the samples which were removed from the dolphins were as follows.

A reasonably clean and wind-free area on the work deck was located immediately after the entire purse seine operation from a particular set was completed. The dead dolphin to be sampled was laid in this area and, depending on the tissue types desired, was handled in a number of ways. If all tissue types were required from one animal the following procedures were undertaken. If only some subset of these tissues were desired, irrelevant procedures were eliminated.

Throughout the entire time we were actively working with the accidentally killed dolphins, we both wore sterile masks across our mouth and nose in order to reduce the possibility of contamination from our normal ventilation. Between handling each dolphin, our hands were thoroughly cleansed in sea water, on board ship distilled water, and then liberally sprayed and rinsed with ethanol. Between the removal of each tissue sample, our hands were liberally rinsed and sprayed with ethanol in order to again sterilize them. When not actually being used, all our instruments (sterile forceps, scissors, and large dissecting scalpel) were stood on end in a container of ethanol to kill any contaminants that may be on them from the last procedure.

If the animal possessed any debris adhering to its skin from the purse seine operation, the animal was liberally washed in first, sea water, then a

Accession No. D7 Species Stenella longirostris

Date 10/17/79 Set No. 16 Sex ♂ Age JUV Population white belly

Collecting Locality 09·34· " (N) S LAT; 107·29· " W LONG.

Comments Orig. collection # DEL037

Protocols Used:

Med.	Temp.	Ship	Skin	Lung	Kidney	Heart	Cornea	Embryo	Blood			
L-15	Ambient	Air		✓		✓						
L-15	Ambient	Mail	✓									
L-15	Refrig	Air		✓								
L-15	Refrig	Mail		✓								
L-15	Frozen	Air	✓	✓		✓✓						
L-15	Culture		✓	✓	✓							
McCoys	Ambient	Air	✓									
McCoys	Ambient	Mail										
McCoys	Refrig	Air	✓									
McCoys	Refrig	Mail		✓		✓						
McCoys	Frozen	Air										
McCoys	Culture											

Figure 4. An example of a data accession form used to record data on tissue samples removed from accidentally killed dolphins.

small quantity of the onboard distilled water. In cases where skin tissue or corneal tissue were to be removed, only ethanol was used to cleanse the area in the manner described below. Where an incision was being made in the external anatomy of the animal to expose internal organs to be sampled, a soap scrub was used in addition to the ethanol. Soap was not used in cases where skin and cornea were to be removed as the soap frequently will reduce the viability of tissue samples that have been treated with them.

Where applicable, after washing the animal with sea water and on board ship distilled water, a liberal amount of liquid dishwashing detergent was placed on the surface of the animal that was later to be cut and the area was thoroughly scrubbed using a veterinarian sponge scrubber. The area was then thoroughly rinsed with on-board ship distilled water and then sterilized twice by spraying the area with ethanol from a spray bottle. The ethanol was then allowed to dry and the appropriate incisions made.

If a skin sample was desired, usually the area around the dorsal fin or flukes was utilized (see Figure 5). The area from which the skin was to be removed was liberally sprayed with ethanol from a mist spray bottle and scrubbed with a sterile veterinary sponge scrubber in order to dislodge any contaminants which were tightly adhering to the skin. The area was then again liberally sprayed with ethanol in the above manner. The ethanol was allowed to dry and then a small section of the fin was firmly grasped with a pair of sterile tweezers which had been soaking in the container of ethanol mentioned above. Scissors or a scalpel (which were also being soaked in the ethanol container) were used to excise a small piece of skin approximately 2 to 3 mm. x 5 to 7 mm. x 7 to 10 mm. in such a way so as to obtain pink tissue. The samples were immediately placed in a vial of prepared medium

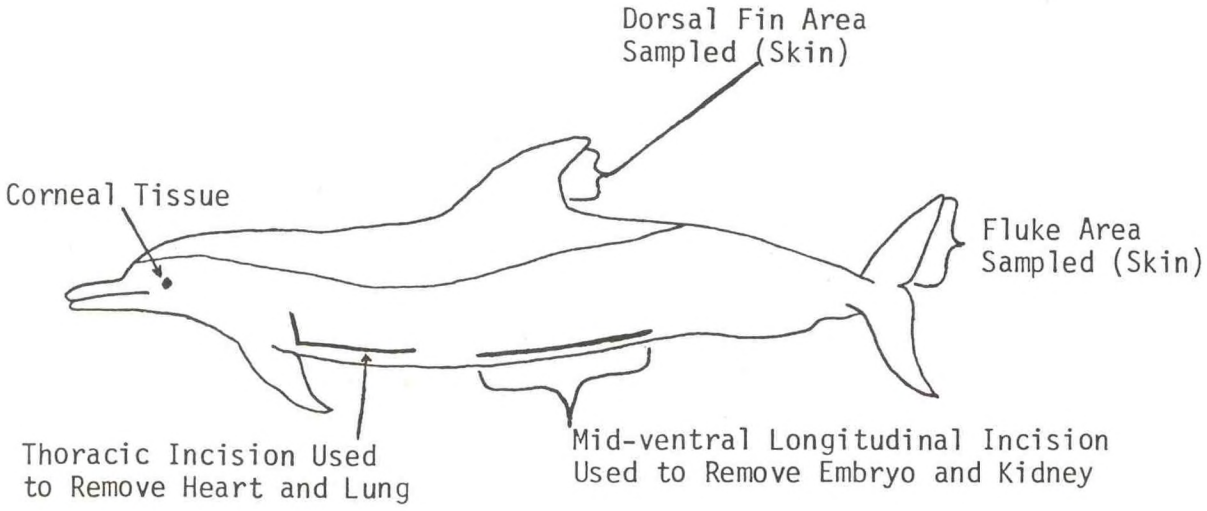


Figure 5. Diagram of incisions made (and external tissues used) to remove tissue samples from accidentally killed dolphins.

which was uncapped, only long enough to physically drop the tissue sample in, and then recapped.

To obtain lung or heart tissue a longitudinal slit was made on the left side of the animal in the immediate area of the flipper for approximately 8 to 12 inches and another incision vertically from the anterior edge of the longitudinal cut to extend dorsally approximately 4 to 6 inches (such vertical cuts were made between the ribs--see Figure 5). The ribs and connecting tissue in the area were then grasped and pulled laterally and dorsally out of the way such that the heart and lung tissues were exposed. When a blood sample was desired, a 10 ml. heparinized sterile syringe was used to puncture one or both of the ventricular cavities of the heart and to draw blood. Next, lung tissue was removed in a similar manner to that described for skin, by grasping a small section of lung with the tweezers and excising it with the scissors or scalpel. Heart tissue (being careful to obtain a sample near the aorta--as tissue from this area has the highest growth potential) was gathered in the same manner. Both heart and lung were placed in individual vials of medium in a manner similar to that described for skin, above.

In order to remove kidney tissue and in the case of females, check for, and remove if necessary, embryonic tissue; the ventral surface of the animal was scrubbed with soap, and cleansed with ethanol in the general method described above. A mid-ventral longitudinal incision was made in the abdominal wall beginning near the middle of the animal and running posteriorly for approximately 12 to 18 inches (see Figure 5). We then grasped the abdominal wall and pulled it away so as to expose the internal organs. Using the ethanol-soaked sterile forceps, we probed in the visceral cavity until we could determine whether or not an embryo was present. If an

embryo was present, a longitudinal incision was made in the lateral wall of the uterus of a sufficient length to release the embryo. The embryo was transferred directly to an ethanol-sterilized container (in this case, a sterilized stainless steel emesis pan). Appropriate tissues (those that we have found to grow the best for embryos are heart, lung and limb--or flipper and fluke--buds) were removed from the embryo and placed in the vial in the manner mentioned above. No attempt was made to sterilize the embryo with alcohol prior to tissue removal unless it was felt that some contamination occurred by the embryo touching a contaminated item as it was being removed from the uterus. The heart and lung tissue was removed from the embryo by making incisions in the same manner as that listed for adults, only scaling the operation down in size. On small embryos, the entire heart and lung were removed, cut into pieces approximately the size listed above for skin, and placed in sterile vials. On larger embryos (which we did not obtain in our actual sample) we would have removed only a small portion of the internal organs as we do for adults. On the embryo that we obtained, we removed both flippers and both tail flukes, cut them into the appropriate size pieces, and placed them in sterile medium vials. Had the embryo been larger, only a portion of the fluke and flipper would have been removed.

Kidney tissue was removed by grasping a small section of the kidney with the sterilized forceps and excising it with a pair of scissors or a scalpel, and placing the sample in a sterile medium vial. In order to remove cornea, the entire area around the eye needed to be cleansed with ethanol only (again in this case we did not use soap so as not to reduce the viability of the collected tissues). The ethanol procedures are the same as those listed for skin. First, we made a longitudinal incision in the posterior and anterior corner of the eyelid, each running approximately one inch in length away from

the corner of the eye. We then grasped the eyelid with the forceps and using a scalpel, excised a large portion of the eyelid and discarded it in order to better expose the eye. We then again sprayed the surface of the eye with ethanol and allowed it to dry for reasons of sterilization. Next, we grasped the edge of the cornea with the sterile forceps and gently excised the cornea from the eye using a scalpel. The entire cornea was placed intact in a sterilized medium vial.

After the tissues were gathered, they were stored on board ship in the manner described in section 4.0 below. No attempt was made to remove every tissue type or utilize every treatment for every animal, as the resultant number of vials for us to work with in the laboratory would have been prohibitive. We, instead, attempted to assure that each tissue type and each treatment was gathered on a representative sampling in order to test the viability of samples and the effectiveness of treatments. Appendix B shows copies of each of the accession forms used while on board ship indicating the tissue types and treatments which were employed for each animal.

3.3 Results

A total of 248 tissue samples of various kinds were extracted from the 16 animals mentioned above. Of these 248 samples, 208 were of non-frozen tissue and 40 were of cryogenically frozen material. One hundred and seventy-two of the samples were of the northern offshore *Stenella attenuata* and 76 samples were of the eastern *Stenella longirostris*.

All the tissue types (skin, lung, kidney, heart, cornea and embryo) which we intended to test were well represented by samples; however, more lung and heart were collected than anything else. A complete breakdown of

the tissue types obtained, grouped according to species, and population (stock), tissue type, and individual animal are summarized in Table 2.

Table 2. Dolphin tissues obtained on the M/V Maria C. J. during cruise No. 565 (October-November, 1979)

Grouped According to	Group	Non-Frozen Samples	Frozen Samples	Total Samples
Species & Population	S. attenuata (Northern Offshore)	146	26	172
	S. longirostris (Eastern)	62	14	76
Tissue Type	Skin	31	8	39
	Lung	49	8	57
	Kidney	34	7	41
	Heart	57	13	70
	Cornea	16	2	18
	Embryo	21	2	23
Individual (by dolphin accession number)	D1	40	5	45
	D2	11	3	14
	D3	3	1	4
	D4	3	1	4
	D5	2	1	3
	D6	11	2	13
	D7	9	4	13
	D8	4	1	5
	D9	2	1	3
	D10	2	1	3
	D11	2	0	2
	D12	1	0	1
	D13	13	4	17
	D14	35	5	40
	D15	50	9	59
	D17	20	2	22
Totals		208	40	248

4.0 Analysis of Tissue Storage and Viability on Board the Tuna Vessel

4.1 Materials and Methods

Prior to my departure for the cruise on the tuna vessel, we carefully worked out in our laboratory the protocols to be used both in tissue removal and in storage of tissue on board the tuna vessel. This was done in an attempt to gather as many different types of tissues to test as was feasible, and also to guarantee obtaining a sampling of tissue from at least several animals. An accession form was constructed indicating on one axis the tissue types to be removed from the animals and on the other axis the treatments which were to be used for each tissue sample (see Figure 4).

I had assumed a priori that certain tissue types and certain storage techniques would be superior to others. Embryonic tissues are by far the fastest growing tissues (in culture) for most animals, but they do have the limitation of not remaining viable in storage for long periods of time. Both of these factors are a function of the associated high rate of cell division. Skin, which is a very slow growing tissue, was predicted to be the most viable tissue type on board the research vessel because of its slow growth rate and therefore its longevity as a viable storage item. Cornea is also a very slow-growing tissue and has some of the same characteristics. Lung, kidney, and heart are mid-range tissues whose growth and storage characteristics fall somewhere between embryo and the skin and cornea group. Using the protocols that were developed a priori to embarking on the tuna seiner, a variety of tissues were removed from the dolphins collected (including heart, aorta, lung, kidney, cornea, skin, and embryo). These tissues were stored under various protocols designed to determine the best

method of obtaining, storing, and transporting living viable tissue for culture at our laboratory.

The M/V Maria C. J. has a variety of storage facilities in which to store dolphin tissue. As we were trying to simulate conditions which would be encountered by standard NMFS observers, we used storage facilities that would generally be available to any observer on board a tuna seining vessel. The two major storage protocols tested which relate directly to the ship's conditions were ambient temperature and refrigerated samples. The M/V Maria C. J. (as I understand is common practice for most tuna vessels) is air-conditioned and has walk-in refrigerator and walk-in freezer storage compartments. The ambient temperature samples were held at ambient air-conditioned room temperature (averaging about 21.0°C) in the cabin in which I lodged during my stay on board the vessel. The refrigerated samples were held in a small refrigerator (4.0°C) in the Chief Engineer's cabin. The temperature in the refrigeration unit was checked periodically against the temperature in the walk-in refrigerator and the two units were constantly within approximately one degree centigrade of each other indicating no real difference between the two storage sites. No tissues were placed in the walk-in freezer storage compartments, as freezing of cells unless cryogenically done destroys them. We did, however, attempt cryogenic storage of a sample of cells following the basic techniques of Claflin, et al. (1978) by the use of a liquid nitrogen container which we brought on board. This type of storage would be impractical for NMFS observers to employ as a result of the prohibitive cost of the tanks, the short-lived nature of the liquid nitrogen, and the special handling and chemicals that would be necessary for this type of storage. The results of the various storage protocols are discussed in Section 4.2 below.

In addition to those listed above, two other protocols were tried on board ship in an attempt to assure adequate tissue sampling from the dolphins. Both of these procedures (described below) would be impractical for NMFS observers to attempt as a result of the cost of the equipment involved and the training necessary for them to be able to participate in the associated work.

The first of these involved setting up cultures in much the same methods as those described in Section 5.1, below, using a small portable sterile hood set up in the Chief Engineer's room. These samples were then placed in a small incubator (which I kept in my living quarters on board the ship), continually incubated until I returned to port on the M/V Elizabeth C. J., and were transported via insulated containers to Logan. The other protocol attempted was that of using dolphin blood to make usable chromosome spreads for the dolphins harvested. The techniques used to remove and process the blood are described in Section 4.3, below.

I also tested whether McCoys 5a medium (modified), requiring pH control, or Leibovitz's L-15 medium ("L-15"), which does not need CO_2 for pH control, is best for the storage and transportation phase. I tested each tissue type sampled in both media at both temperatures. Close attention was given to possible interspecies differences in such medium requirements. All test media contained 20 percent fetal calf serum, 20.8 mcg/ml Gentamycin (a general and highly versatile antibiotic of outstanding temporal viability) and 2.08 mcg/ml Fungazone (a general fungacide--Amphotericin-B--standardly used in tissue culture work). A series of samples were also analyzed to determine the maximum time that tissues could be effectively stored prior to culture.

4.2 Results

Relatively few of the samples gathered while on board the M/V Maria C. J. suffered any form of contamination (see Table 3). A total of 12 samples (from a total of 208 non-frozen samples) suffered gross fungal or bacterial contamination and necessitated the discarding of the sample. This constituted only 5.8 percent of the entire non-frozen sample. An additional 21 samples were mildly bacterially contaminated, but standard cleansing procedures and enriched antibiotics used in the laboratory successfully eliminated the contamination in all cases. The bacterially contaminated samples constituted only 10.0 percent of the entire non-frozen samples, and when lumped with the grossly contaminated and discarded samples, constituted only 15.9 percent of the entire non-frozen sample population. These figures are in no way alarming, especially considering the asterile environment on board a tuna seiner. We would expect that a slightly higher percentage of contaminated samples would be received by us the second year with NMFS observers gathering the tissue samples.

The best index of viability of stored tissues on board the research vessel was the analyses of the growth of the tissues when cultured in our laboratory. These data are summarized in Table 3. Our analyses indicate that the choice of medium (McCoys or L-15) made a difference in the viability of the tissue samples. Approximately 6% of the samples stored in L-15 medium survived to produce viable tissues, but a total of 14% of the tissues stored in McCoys produced viable tissue. When lung alone is analyzed, the observed difference is not as dramatic: in L-15 approximately 22% of the lung survived and in McCoys approximately 36% survived. We did find, however, that some extremely slow growing tissues (skin and cornea) maintained better in L-15 medium, which requires no pH control. Several of

Table 3. Dolphin tissue storage viability on the M/V Maria C. J. during cruise no. 565 (October-November, 1979)

Analysis by	Treatment	No. of vials without obvious contamination	No. of mildly contaminated vials	No. of severely contaminated vials discarded	Total no. of vials established	Total no. of vials that produced viable tissue	Percentage of vials established that produced viable tissue
Medium	L-15	85	11	8	96	6	6.2
	McCoys	90	10	4	100	14	14.0
Temp.	Ambient	85	10	11	95	8	8.4
	Refrigerated	90	11	1	101	12	11.9
Species	S. attenuata	128	10	8	138	16	11.6
	S. longirostris	47	11	4	58	4	6.9
Tissue Type	Skin	25	3	3	28	0	0.0
	Lung	42	5	2	47	14	29.8
	Kidney	28	5	1	33	0	0.0
	Heart	47	6	4	53	0	0.0
	Cornea	16	0	0	16	1	6.2
	Embryo*	17	2	2	19	5	26.3
Time until set up (lung only)**	Immediately***	2	0	0	2	2	100.0
	2 weeks-4 weeks	3	2	0	5	3	60.0
	4 weeks-6 weeks	15	3	0	18	11	61.1
	6 weeks-10 weeks	1	0	0	1	1****	100.0
	10 weeks-14 weeks	1	0	0	1	0****	0.0
TOTALS		175	21	12	196	20	10.2

*Consisting of pieces of embryonic heart, lung, and flipper and fluke buds.
**Dolphin D14, D15, and D17 excluded as so many samples were taken as to severely skew the results.
***Samples set up "immediately" were incubated on board ship and are not counted in any other totals.
****This sample was held back as a test (see text for details).

the samples of skin and cornea stored in McCoys became too alkaline (particularly those that were stored under refrigeration).

In general, tissues stored under refrigeration fared better than tissues stored at ambient temperatures (11.9% for refrigerated samples and 8.4% for ambient). A variety of reasons would be responsible for this relationship but the two most outstanding would be that at lower temperatures bacterial and fungal growth would be greatly retarded (such that we could interact in our laboratory with any form of contamination), and that the growth of the tissue itself was retarded such that the nutrients in the medium were not as quickly depleted.

Our figures indicate that tissue from Stenella attenuata fared somewhat better (11.6% viable) than the tissue from Stenella longirostris (6.9% viable). These figures may, however, be somewhat misleading inasmuch as they include figures for the embryo from D15 (S. attenuata).

The most striking difference encountered in any of the storage methods which we tested was the differential viability of tissue types on culturing them. Table 3 shows that lung and embryo fared far better than any other tissue when cultured. Both of these tissues survived to produce between 25% and 30% viable tissue cultures. All the skin, kidney, and heart tissue cultures failed to grow. Cornea, which produced approximately 6% viable tissue, never grew to the point of producing a monolayer culture that was in any way utilizable for analysis in our laboratory. Therefore, the only tissues which consistently survived storage on board the research vessel were embryo (more specifically, portions of the lungs, heart, and flipper and fluke buds) and adult lung tissues.

We had initially intended to utilize two different shipment techniques (cultures to be returned on the airplane with me and cultures to be mailed

via the fastest air mail service) but as a result of the low number of dolphins obtained, I decided to eliminate this variable from our analysis in favor of obtaining a larger number of viable specimens in our laboratory. Treatments on the data sheet (Figure 4) listed with similar medium and temperature regimen but different shipment regimen were handled in an identical manner.

4.3 Special Testing of Blood Culture on Board the Tuna Vessel

4.3.1 Materials and Methods

Blood was drawn from each of the killed dolphins in the manner described in Section 3.2, above. Three 15-ml centrifuge tubes were established for each animal with the following solution: 10 ml of mixed medium (containing 20% fetal calf serum and 20.8 mcg/ml Gentamycin and 1% phytohemagglutinin). In each of the three tubes 1/2 ml of blood was added. The tube was recapped and inverted gently several times to thoroughly mix the blood into the medium. The blood was incubated at 37°C for 71 hours in the incubator in which tissue culture samples were also kept. The tubes were inverted gently twice a day to thoroughly mix the blood into the medium. At 71 hours from the inception of incubation, the tubes were removed from the incubator and treated with 4 drops of Colcemid solution, and again placed in the incubator for 1 hour. At the end of the hour (which totals 72 hours from the inception of incubation), the tubes were centrifuged for 5 minutes and the supernatant removed. The cells were then suffused with a 10 ml hypotonic treatment of .075 molar KCl, and the solution thoroughly mixed by bubbling with a pipette. The hypotonic treatment was left on the cells for 20 minutes and the tubes were then again centrifuged for 5 minutes, and the supernatant removed. A fixative

(consisting of 3 parts methanol to 1 part glacial acetic acid) was gently added to the top of the cell pellet. After approximately 3 ml of fix had been added to the centrifuge tube, the tube was allowed to stand for approximately 10 minutes. Cells in the bottom of the tube were then mixed in the fixative by bubbling them gently with a pipette. Five ml of fresh fix was then added, and the entire solution again agitated gently by bubbling with the pipette.

Each of the tubes was centrifuged for a period of 5 minutes and the supernatant removed. The fixative treatment was repeated (omitting the 10-minute wait after the first addition) as many times as was necessary until the majority of red blood cells had been removed from the solution and the remaining white blood cells looked as if they were properly fixed and ready to make slides.

As no microscope was on board with which I could analyze any chromosome spreads or conduct any banding protocols, the prepared tissues were refrigerated until I returned them to our laboratory in Logan where slides were made following the same protocols listed in Section 6.1, below. The banded and prepared slides were analyzed for usability in our laboratory.

4.3.2 Results

None of the blood samples which were run produced chromosome spreads suitable for banding analysis in our laboratory. The most probable reason for this failure is the temperature regimen under which the cells were held in the incubator. Every attempt was made to maintain the incubator temperature at a constant 37°C; however, as a result of fluctuation of the power on board ship, it was difficult for me to control the temperature any

closer than from about 33.8° to 37.1°. This fluctuation may have been too much for the blood cells (which are less resistant than fibroblasts in tissue culture) to withstand, and probably resulted in their demise.

5.0 Analysis of Growth Potential of Tissues Received in the Biomedical Laboratory

5.1 Materials and Methods

The materials and methods for assessing the growth potential of tissues are the same as the first set of steps in the cytogenetic analysis. They will be listed here and shall not be repeated in Section 6.1, below.

Once in our laboratory, tissue pieces were minced into tiny fragments in a sterile petri plate with a sterile blade. This process was repeated until the fragments were cut as small as possible using this technique, most of them ranging in size from approximately 0.01 to 0.1mm in diameter. The fragments were then placed inside a culture flask with a sterile needle, allowed to attach by drying for a brief time, and then carefully flooded with enough growth medium to completely cover the fragments. The growth media used were the same as those used for tissue storage (L-15 or McCoys 5A), but with antibiotics added in addition to the standard Gentamycin and Fungazone where any signs of contamination were evident. The additional antibiotics used were penicillin, streptomycin, and Tetracycline as prepared for human intermuscular injections, but in predetermined concentrations suitable for tissue culture use.

The culture flasks were incubated at 37°C until sufficient cell growth occurred as a monolayer out from the fragment. At this stage, the cells were removed by washing with a trypsin solution and transferred to a new flask with fresh medium. Once transferred, the cells were allowed to grow to fill the new flask and then trypsinized off and split into two flasks. By this means, as many flasks of cells were obtained as needed. Once

monolayer cultures were obtained, cells were harvested for chromosome analyses.

5.2 Results

As in the analysis of tissue storage and viability on board the tuna vessel, the best index of growth potential of tissues received in our laboratory is the number of viable tissue cultures that grew. In addition, in our laboratory we are able to also assess other parameters such as growth rate, number of secondary flasks produced, number of surplus cultures which have been cryogenically frozen, and more subjectively, the general health profile and viability of the cultures from a particular sample.

Tissues in our laboratory grew in either McCoys or L-15 medium but growth rate was fastest and the viability of the cultures seemed highest when we used McCoys 5A with 20% Fetal Calf Serum and standard antibiotics. After a few weeks of growing tissue in both media, it was felt advisable to switch all the cultures then on L-15 to McCoys. When this was done, the growth rate in the flasks that had formerly been in L-15 increased and the cultures generally seemed to be healthier.

Table 3, presented in Section 4.2, indicates the viability and growth potential of tissues which we received in our laboratory. Slightly over 10% of all the tissue samples established in culture grew. This figure is deceptively low, however, because this percentage is comprised of the total of all tissue types tested. Inasmuch as the majority of tissue types did not grow at all, but are still averaged in with the two tissue types which showed good growth, the total percentage of tissues that grew is low. It should be noted that lung and embryo both produced a high percentage

(between 25 and 30%) of viable cultures, and that no other tissue type produced any significant results in culture. The only growth observed other than that for lung and embryo was for one sample of cornea which produced a small colony of cells in a primary flask, but was far too small and slow growing to be able to harvest with any success. Additionally, skin tissue routinely generated a few epithelial cells (unsuitable for chromosome analysis) but consistently failed to produce any fibroblasts (one of the tissue types used in chromosomal analysis).

The growth rate and viability of the embryonic tissue which we obtained was far better than the growth rate or viability for any of the lung tissue which was sampled. However, the lung tissue seemed consistent with other tissues sampled from other types of animals which we routinely grow in our laboratory. An index of this viability is in the tissues which we cryogenically froze for future use. Six of the 14 samples which we froze for future use were of embryonic tissue, the remaining eight were from lung tissue from three other animals.

The length of time between the date the dolphin was killed and the date the tissues were established in cultures seemed to affect the viability of the tissue sample. The two lung tissue cultures which were established while I was on board the ship both grew. These were established within 24 hours of the death of the animal. Tissue samples established from two to four weeks and from four to six weeks from the death of the animal (see Table 3 in Section 4.2) both showed an approximately 60% viability. One tissue sample from D7 known to be a viable sample was split into three separate groups. One of these was established in the 2-4 week date range, another in the 6-10 week date range as a test, and the last in the 10-14 week date range. The sample established in the 2-4 week range

survived and is included with those totals. The sample in the 6-10 week date range survived showing a 100% survivability for that period. The sample that was saved to be established during the 10-14 week period appeared grayish and necrotic when established and did not produce any tissue growth. From these analyses, it would appear that the faster the tissue can be established, the more viable it will be; but that tissue samples retained up to 10 or so weeks may remain viable in culture media.

None of the cryogenically frozen tissue samples produced any viable tissue cultures and this technique (because of the cost, inconvenience, special training necessary for the observers, and lack of success) does not appear to be a fruitful approach to dolphin chromosomal analyses.

6.0 Cytogenetic Analysis of Tissue Samples

6.1 Materials and Methods

Monolayer cultures were harvested for chromosomal analyses during the first few passages to reduce the possibility of culture dependent chromosomal variation. In harvesting cells from the cultures, standard procedures of blocking mitosis at metaphase with colcemid for 1 hour, removing the cells with trypsin, and subjecting the cells to a 20-minute hypotonic treatment (3 parts distilled water to 1 part McCoys 5a medium) were followed. The cells were then fixed for 20 minutes in 1 part glacial acetic acid to 3 parts absolute methanol. The cell mixture was concentrated by centrifugation after fixation, washed with fresh fixative, and dropped onto clean wet microscope slides for speading out the chromosomes. The prepared slides were tipped up and air dried at an 80° (from horizontal) angle, then placed in a 50°C oven overnight prior to G-banding and stored dry at room temperature overnight prior to C-banding or silver staining.

6.1.1 G-banding

The standard trypsin Giemsa method was used employing 6 Coplin jars with the following solutions: (1) working trypsin solution of 1 part .25 percent of trypsin in Hanks' solution (-Ca++ and Mg++) with 5 parts of Hanks' solution (-Ca++ and Mg++); (2) Hanks' solution as is used in the trypsin solution; (3) 70% ethanol; (4) 95% ethanol; (5) 2% Giemsa stain in a .01 M phosphate buffer; (6) distilled water. The slide to be treated for G-banding was immersed in the trypsin solution for 2 minutes, rinsed in Hanks' solution, rinsed in 70% and 95% ethanol, and tipped up and air dried. The slide was then stained in the Giemsa stain solution for 10 minutes, rinsed briefly in distilled water and air dried. After drying, the slide was examined under

an oil immersion lens for band quality; and if good banding was present, representative chromosome spreads were photographed for preparation of a karyotype. If the chromosomes were under or over treated, the duration of the trypsin treatment was adjusted for the next slide. Photographs were cut out to produce G-banded karyotypes.

6.1.2 C-Banding

C-banding was obtained with a modification of the Bull (1978) modification of the barium hydroxide technique of Sumner (1972). In this method, the slide was first treated in a .2N HCl solution for 15 minutes, dipped in a 5% BaOH solution at 46° C, rinsed in .2N HCl and incubated for 15-20 minutes in 60° C 2 X SSC solution; rinsed in 70% and 95% ethanol, and stained 10 minutes in 2% Giemsa stain in .01 M phosphate buffer. The stained slide was rinsed in distilled water, dried, and examined under an oil immersion lens. Suitable metaphase chromosome spreads were photographed. The resulting prints were cut out to produce a C-banded karyotype.

6.1.3 Silver Staining

Silver staining of nucleolar organizer regions (NORs) was accomplished by the method of Bloom and Goodpasture (1976), in which slides were flooded with a 50% aqueous solution of silver nitrate and incubated in a moist chamber at 50° C for 3 hours. The slides were then rinsed, counterstained with 2% Giemsa stain for 3 seconds, and air dried. Chromosome spreads were examined under oil immersion and photographed with the NORs enhanced by use of a green filter. The photographs were either examined directly or a full or partial karyotype was constructed from cut up photographs.

6.2 Results

Cell cultures suitable for cytogenetic analyses were produced on a total

of five animals from the eastern tropical Pacific (see Table 4). Four of these (three males and one female) were Stenella attenuata and one was a male Stenella longirostris. In addition, a cell culture was obtained from a male Stenella plagiodon from the coast of Florida through the American Type Culture Collection. This tissue was from the same animal as that reported by Arnason (1974) as Stenella dubia and later called Stenella plagiodon by the same author (Arnason, 1980). Inasmuch as the karyotype for this species has been published previously (Arnason, 1974), it is used in this study as a basis for comparison.

The karyotypes I constructed generally follow the arrangement introduced by Arnason (1974) without modification. Only one exception exists: the chromosome listed by Arnason as SM9 has been, on the basis of evidence produced in this paper, placed as the last telocentric chromosome (T5). The reason for modification of the Arnason order is covered in Section 6.2.3 (Nucleolar Organizer Regions) and also the Discussion. Although it is not actually a modification of the Arnason order, another minor exception exists: the karyotypes presented for D7, which as a result of a balanced translocation, has the rearranged chromosomes presented at the bottom of the karyotype. Figures 6-11, 13-16, 18-19 show representative examples of G- and C-band karyotypes in the Arnason arrangement with our modification.

6.2.1 G-band Comparisons

The G-band karyotypes for four individuals of Stenella attenuata are shown in Figures 6, 7, 8 and 9. Only one of these animals, D8 (Figure 7) was a female. All chromosome pairs with the exception of two pairs of metacentrics were easily recognizable. Chromosome pairs M2 and M3 were quite

Table 4. Populational, geographical, and temporal data for animals from which successful tissue culture harvests were obtained.

Our Collection Number	Species	Population	Sex	Collection Locality	Date Collected	Original Collection Number
D1	Stenella attenuata	Northern Offshore	M	13°50'N 106°08'W	10/12/79	DEL031
D8	Stenella attenuata	Northern Offshore	F	09°34'N 107°29'W	10/17/79	DEL038
D13	Stenella attenuata	Northern Offshore	M	09°34N 107°29'W	10/17/79	DEL043
D16	Stenella attenuata	Northern Offshore	M	09°38'N 110°00'W	10/25/79	Embryo from DEL045
D7	Stenella longirostris	Eastern	M	09°34'N 107°29'W	10/17/79	DEL037

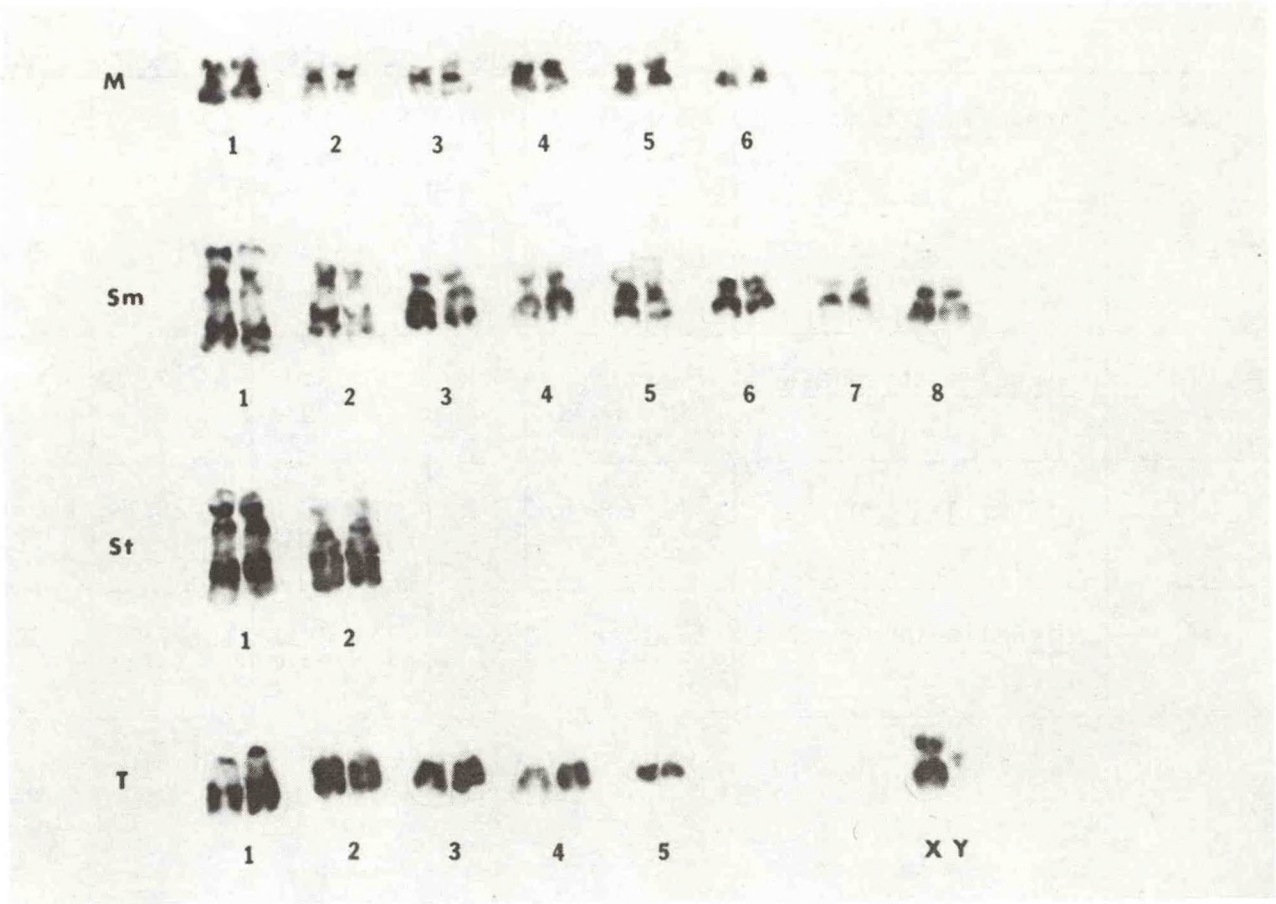


Figure 6. G-banded karyotype of dolphin 1 (male Stenella attenuata).

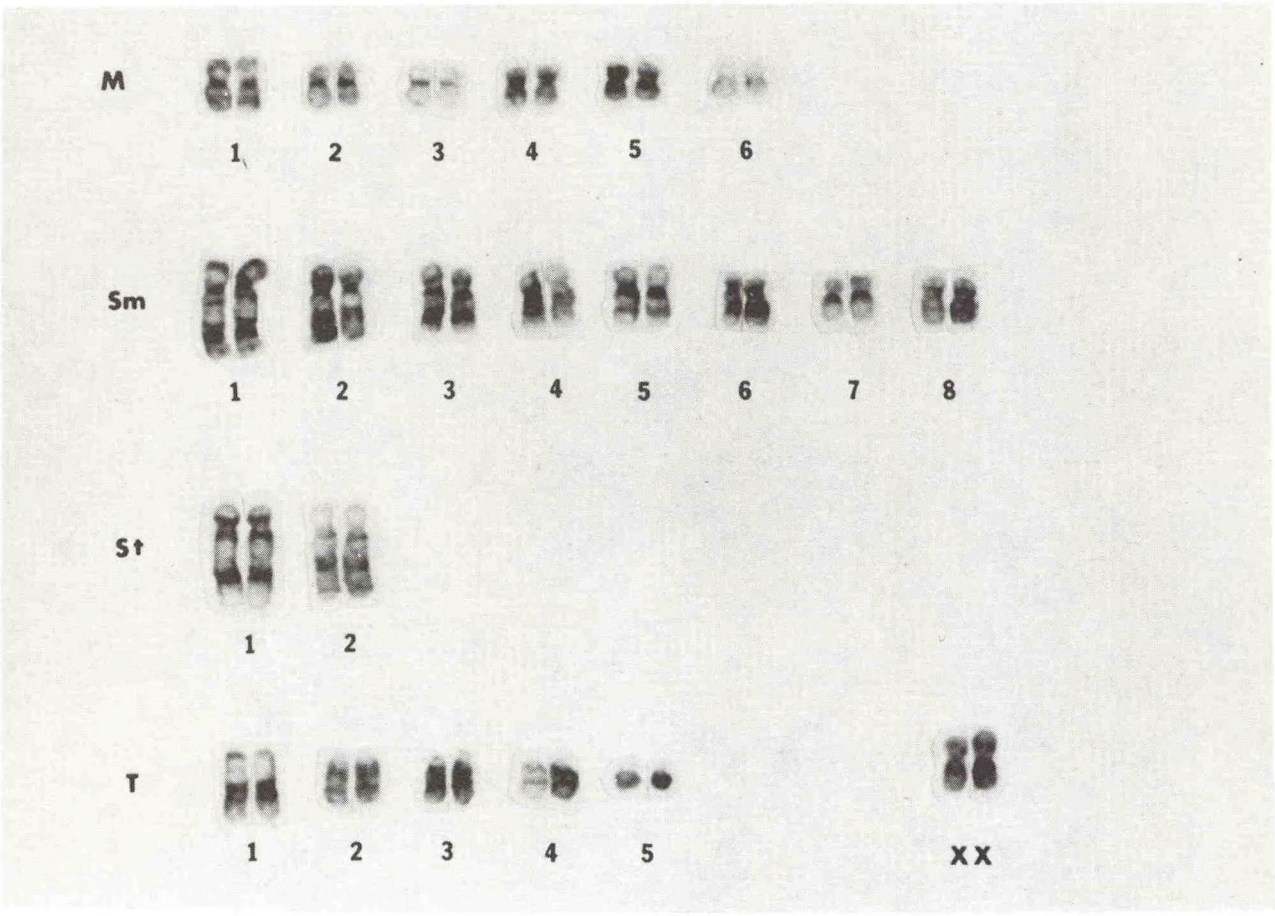


Figure 7. G-banded karyotype of dolphin 8 (female Stenella attenuata).

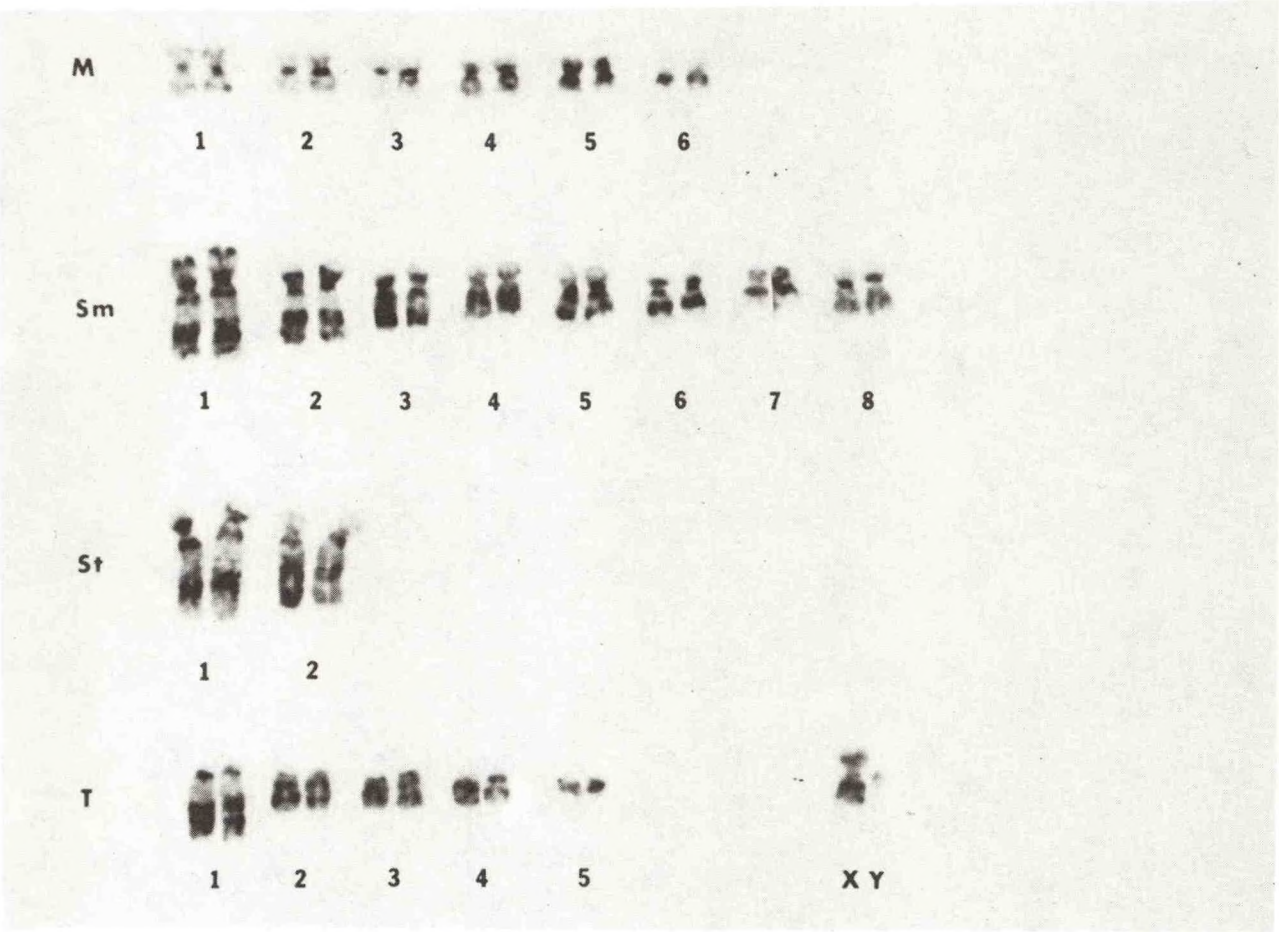


Figure 8. G-banded karyotype of dolphin 13 (male Stenella attenuata).

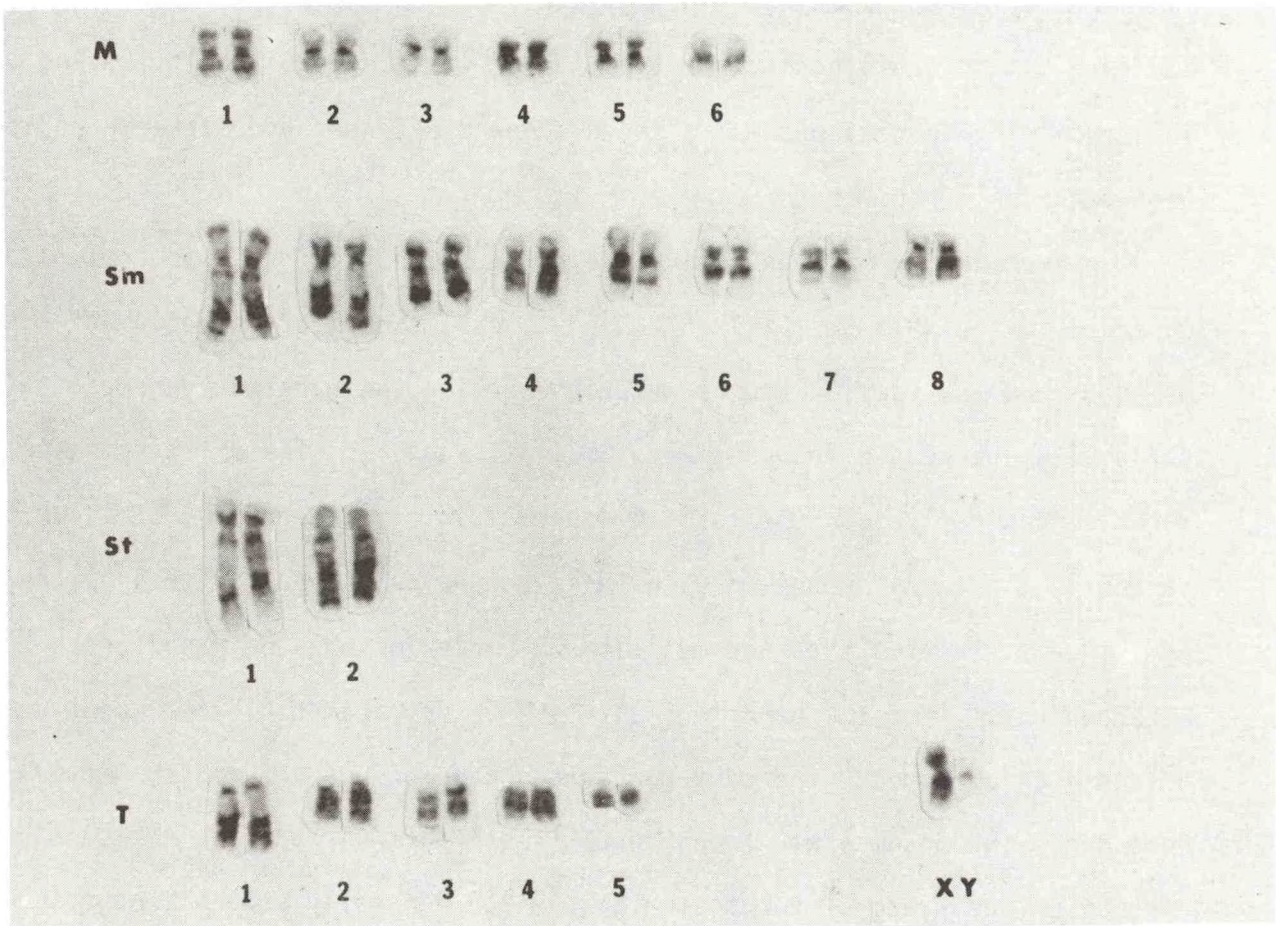


Figure 9. G-banded karyotype of dolphin 16 (male Stenella attenuata).

similar to each other in G-band comparisons as were pairs M4 and M5, and these were separated with much difficulty. Chromosome M2 was separated by its very light but characteristic G-positive band in the mid-region of the q arm. Furthermore, M3 was completely G negative in the p arm whereas M2 occasionally had some slight staining in that region. M5 was distinguished by having a larger distal G-negative area in the q arm and a larger and darker G-positive area near the centromere.

Heteromorphism between individual chromosomes of a pair was commonly observed in the SM1, SM2, ST1, ST2, and T1 chromosomes of Stenella attenuata using G-band analyses. Chromosome pairs showing the greatest degree of variability between homologues were SM2, ST1, and T1. The existing heteromorphism generally took the form of light staining (G-negative) bands which varied in size. A comparison with the C-banded karyotype for each of the individuals revealed that the majority of variation was the result of heteromorphism in the C-band (constitutive heterochromatin) complement on each of these chromosomes. The most pronounced case of heteromorphism between homologous chromosomes was found in D1 (see Figure 6) in which the terminal portion of the p arm of chromosome SM2, which generally stains G-negative, showed no G-negative staining in one of the homologues. Analysis of the C-banded karyotype for the same animal (Figure 13) revealed that the complement of heterochromatin generally found as a terminal block on chromosome SM2 was absent on one of the two homologues. The presence of the G-negative banding region and the conspicuous absence of heterocromatin in the C-band analysis appear to correspond. Similar variation, generally restricted to amount of heterochromatin (or C-positive banding) present, was found in many instances, but was much reduced in relation to the example just given.

G-band analysis confirmed the largest metacentric chromosome to be the X chromosome, and the minute Y chromosome to be the smallest chromosome in the cell.

The G-banded karyotype of Stenella longirostris is virtually identical to that for Stenella attenuata. Visible differences between the karyotypes result basically from the balanced translocation found in the one individual of Stenella longirostris (D7) which was sampled (see Figure 10). This translocation is discussed in detail in Section 6.2.4 below. The information presented in this section ignores the balanced translocation and is handled as if the translocated chromosomes were arranged as are their normal partners. As in Stenella attenuata considerable heteromorphism was found in chromosomes SM1, SM2, ST1, ST2, and T1. The most pronounced heteromorphisms occurred in SM1, SM2, and T1. As with Stenella attenuata, most of the variation occurred in the G-negative staining regions which corresponded to C-positive staining regions in the C-band karyotype.

The G-banded karyotype of Stenella plagiodon (see Figure 11) is quite similar to that described for the two preceding species. Our cell culture for this animal was aneuploid, consistently showing 45 chromosomes. The extra chromosome was identified as a T4. The degree of heteromorphism observed seemed to be less in Stenella plagiodon than in either S. attenuata or S. longirostris. The only major heteromorphisms between individuals of a pair of chromosomes were found in SM2 and T1 which showed similar patterns to the other two species discussed above.

Figure 12 presents a composite G-band karyotype of selected chromosomes from a diploid cell (with one chromosome from each pair represented) for each of the three species discussed above. Analysis of the chromosome comparisons demonstrates complete chromosome banding pattern homology between the three

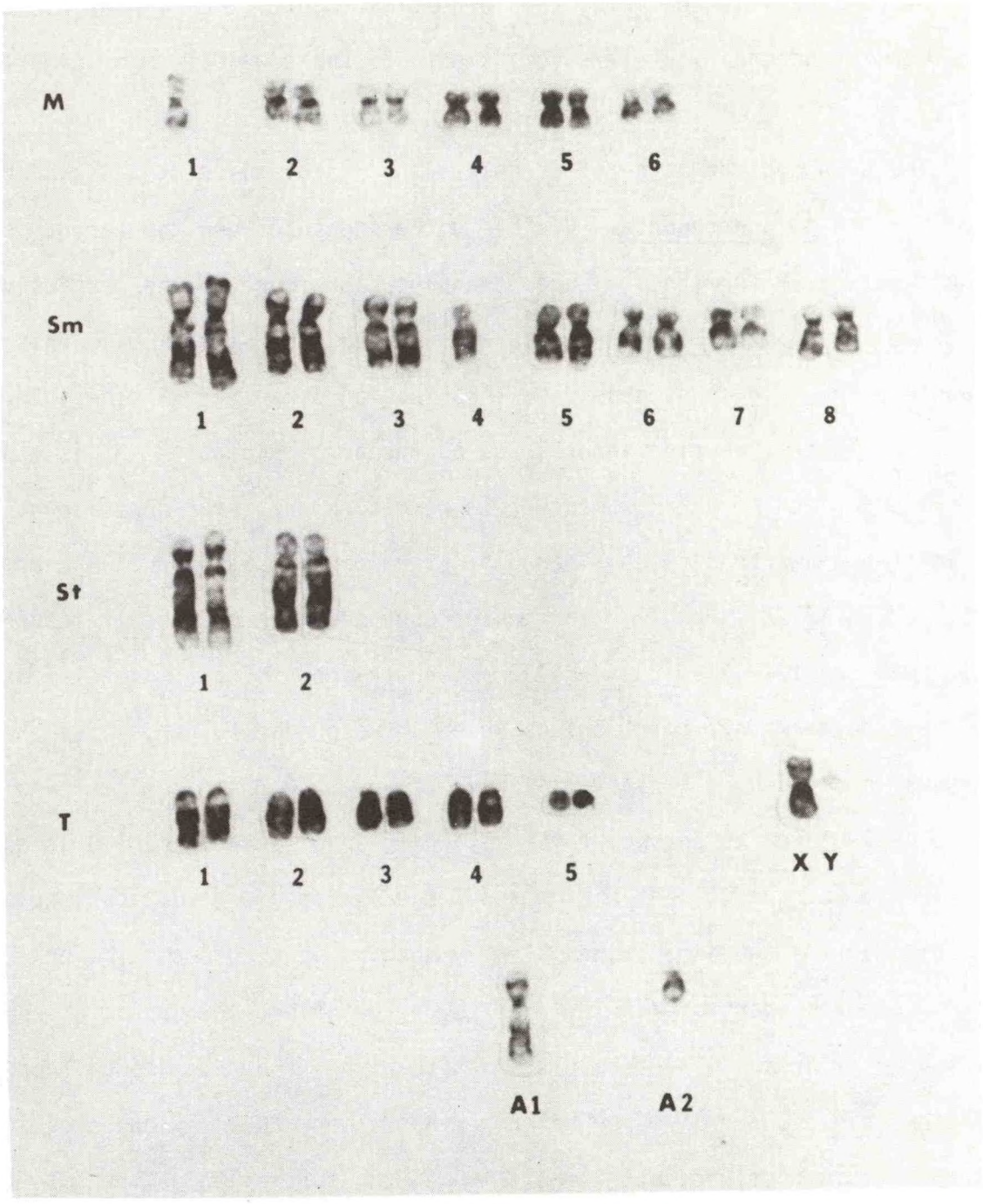


Figure 10. G-banded karyotype of dolphin 7 (male Stenella longirostris).

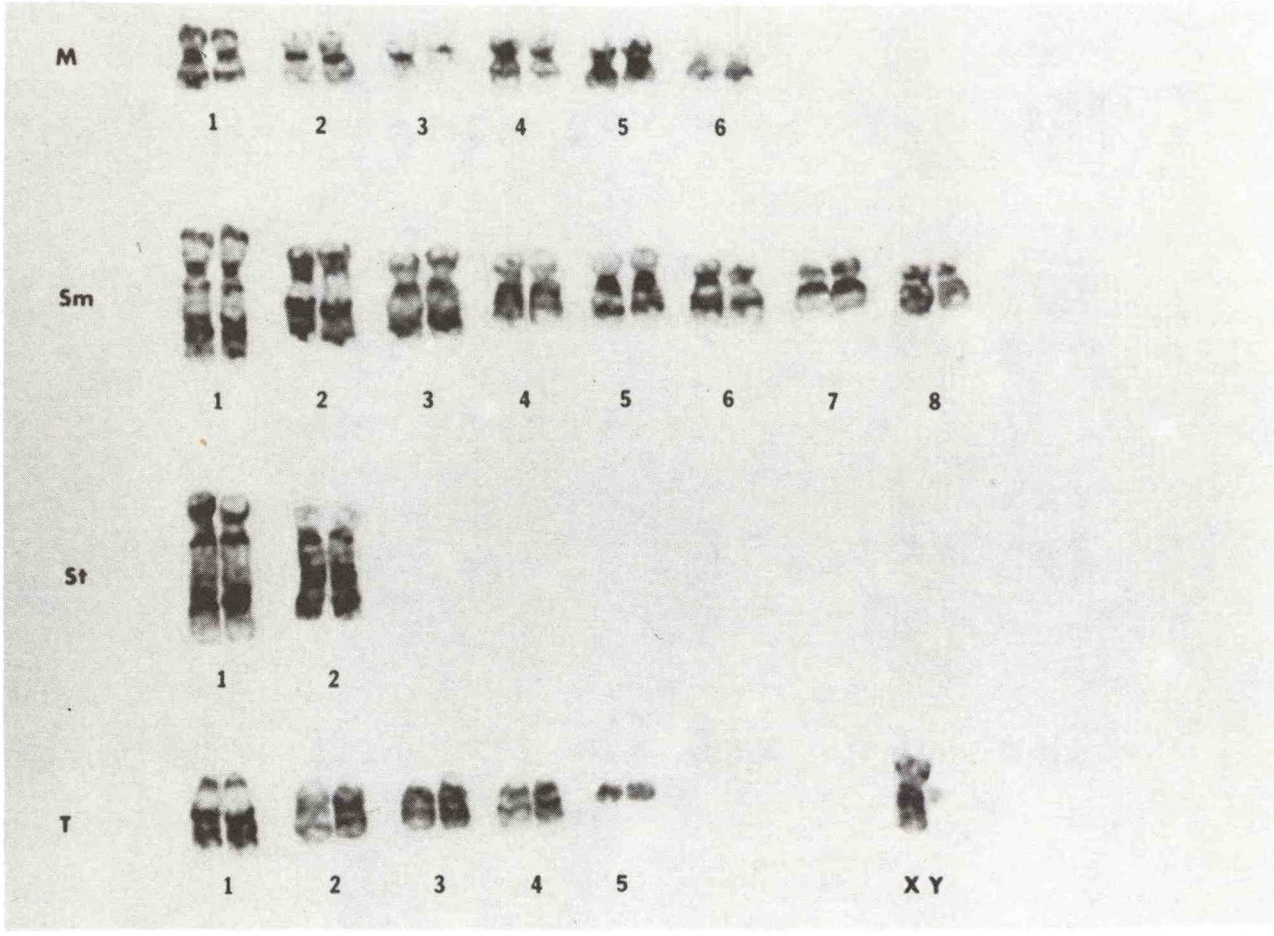


Figure 11. G-banded karyotype of a male Stenella plagiodon.

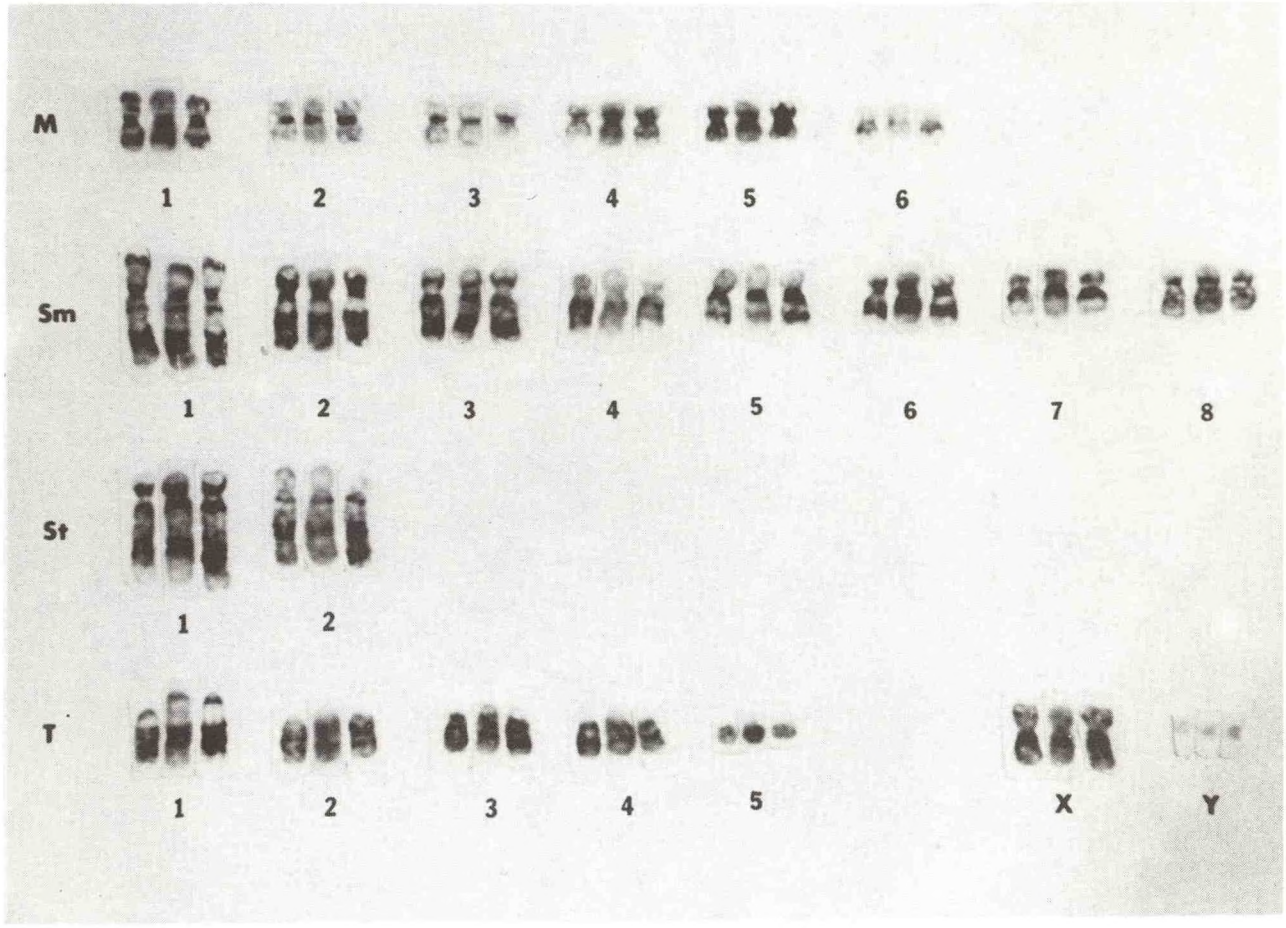


Figure 12. G-banded comparison of selected chromosomes (one from each pair) from diploid cells of three species of Stenella: (from left to right) S. longirostris, S. attenuata, and S. plagiodon.

species under investigation. Some general observations, relating to factors which all three species share in common, can be made as follows. Most of the centromeric or paracentromeric regions on each of the chromosomes are heavily G-positive stained. A notable exception to this observation is found in the centromeric regions of T3 and T4, where very faintly stained (or G-negative) regions exist. Many of the chromosomes (for example SM2 through SM5, inclusive) bear large terminal G-negative blocks on the p arm of the chromosome. Most, but not all, of these G-negative regions correspond to areas of C-positive (constitutive heterochromatin) banding regions in corresponding karyotypes. Minor differences observed in the G-band comparison karyotype result mostly from the heteromorphism mentioned above, and to a lesser extent from the varying photographability of certain chromosomes from particular spreads. Inasmuch as the composite photographs used herein are taken from one cell for each of the species, this variation in photographability is noticeable. The possible danger of confusing photographability with true variation has been eliminated by the use of multiple karyotypes for each animal when cytogenetic interpretations were being made. As a result of space limitations, it is impractical to reproduce these repetitive karyotypes here.

6.2.2 C-band Comparisons

Unlike the extremely stable and conservative G-banded karyotypes presented in the previous section for the three species under investigation, C-banded (constitutive heterochromatin) karyotypes demonstrate a great deal of intraspecific and interspecific variation. With the exception of the variations discussed below, most of the C-banded chromosomes were reasonably similar in nature. Figures 13 through 16, inclusive, show standard C-banded

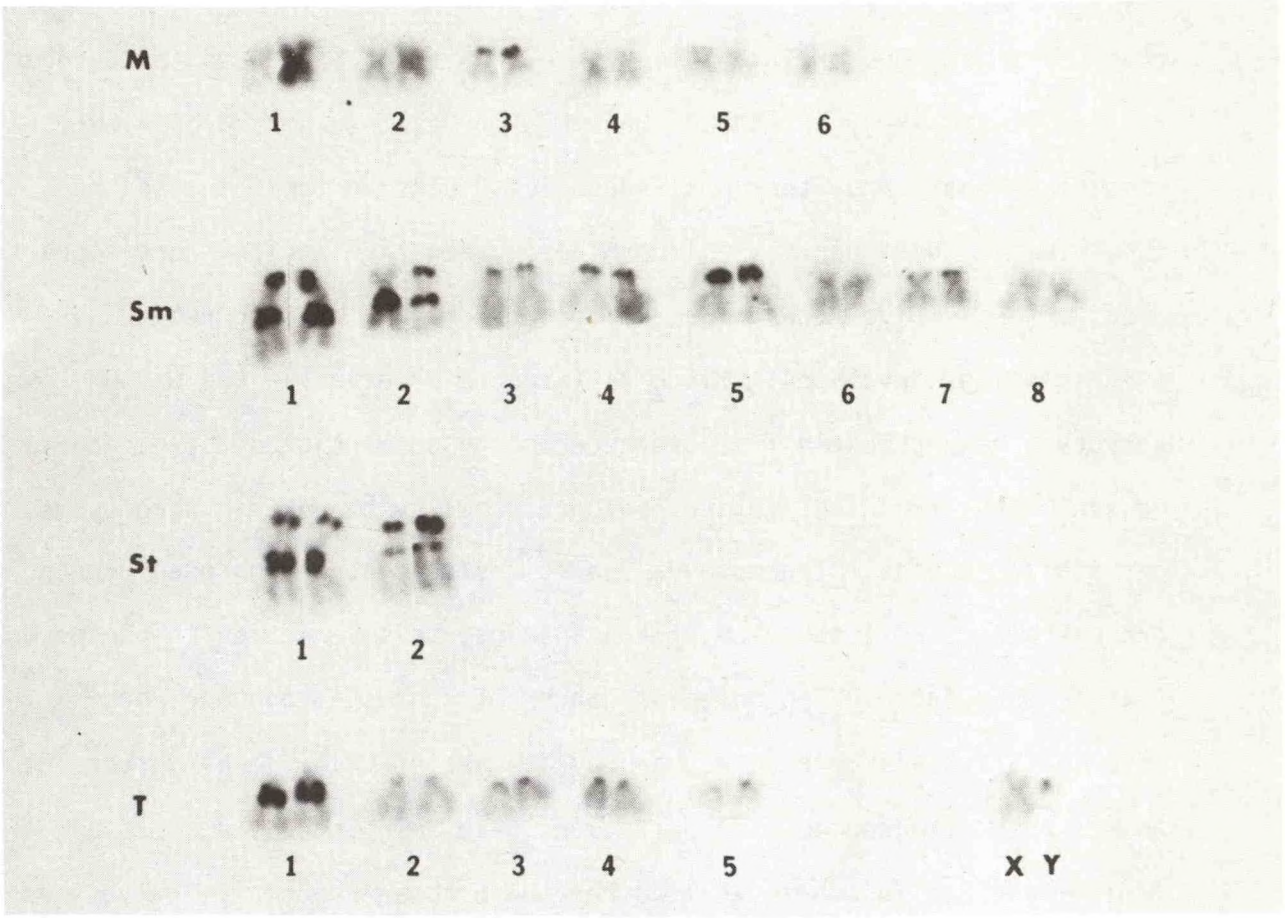


Figure 13. C-banded karyotype of dolphin 1 (male Stenella attenuata).

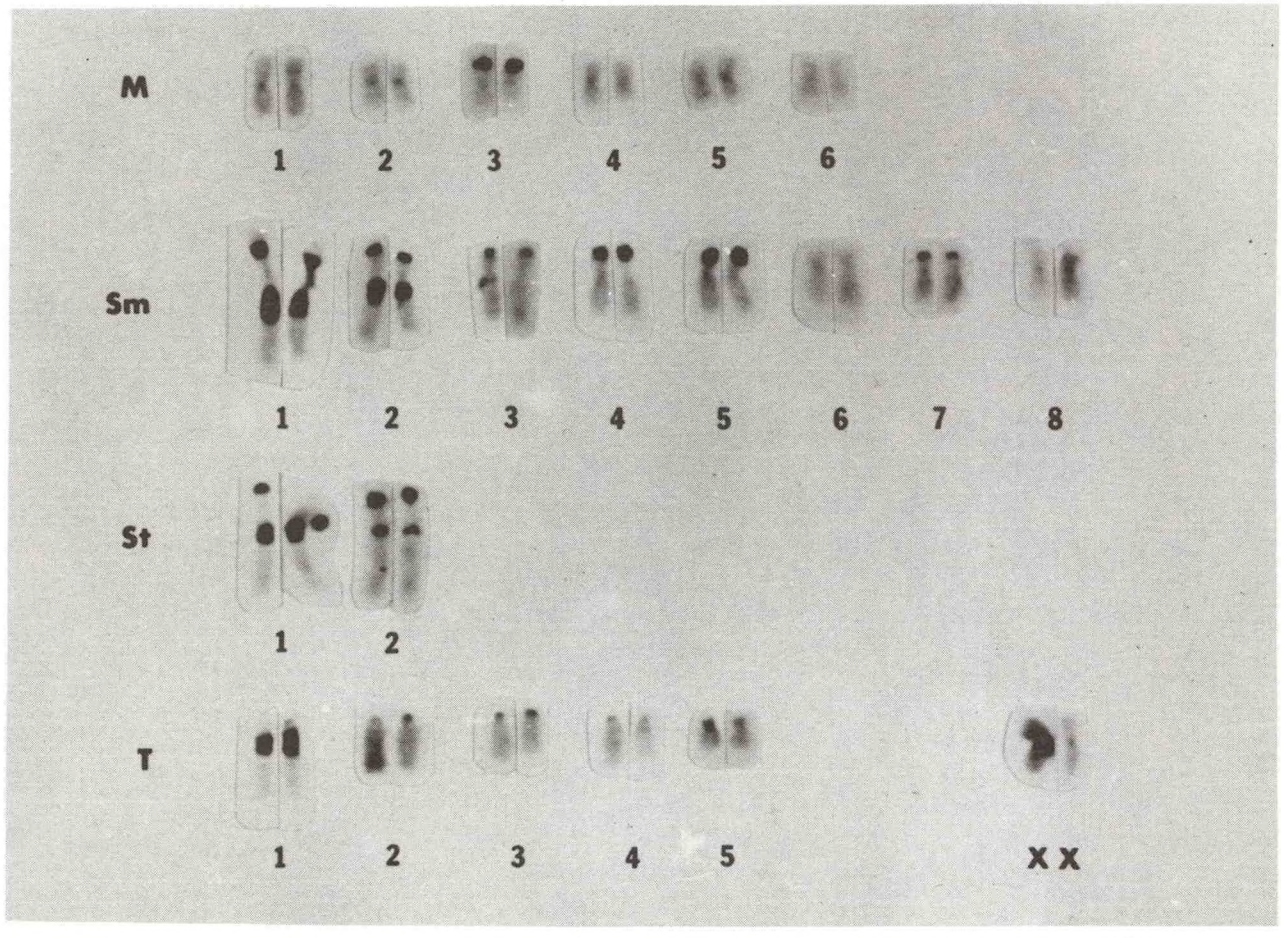


Figure 14. C-banded karyotype of dolphin 8 (female Stenella attenuata).

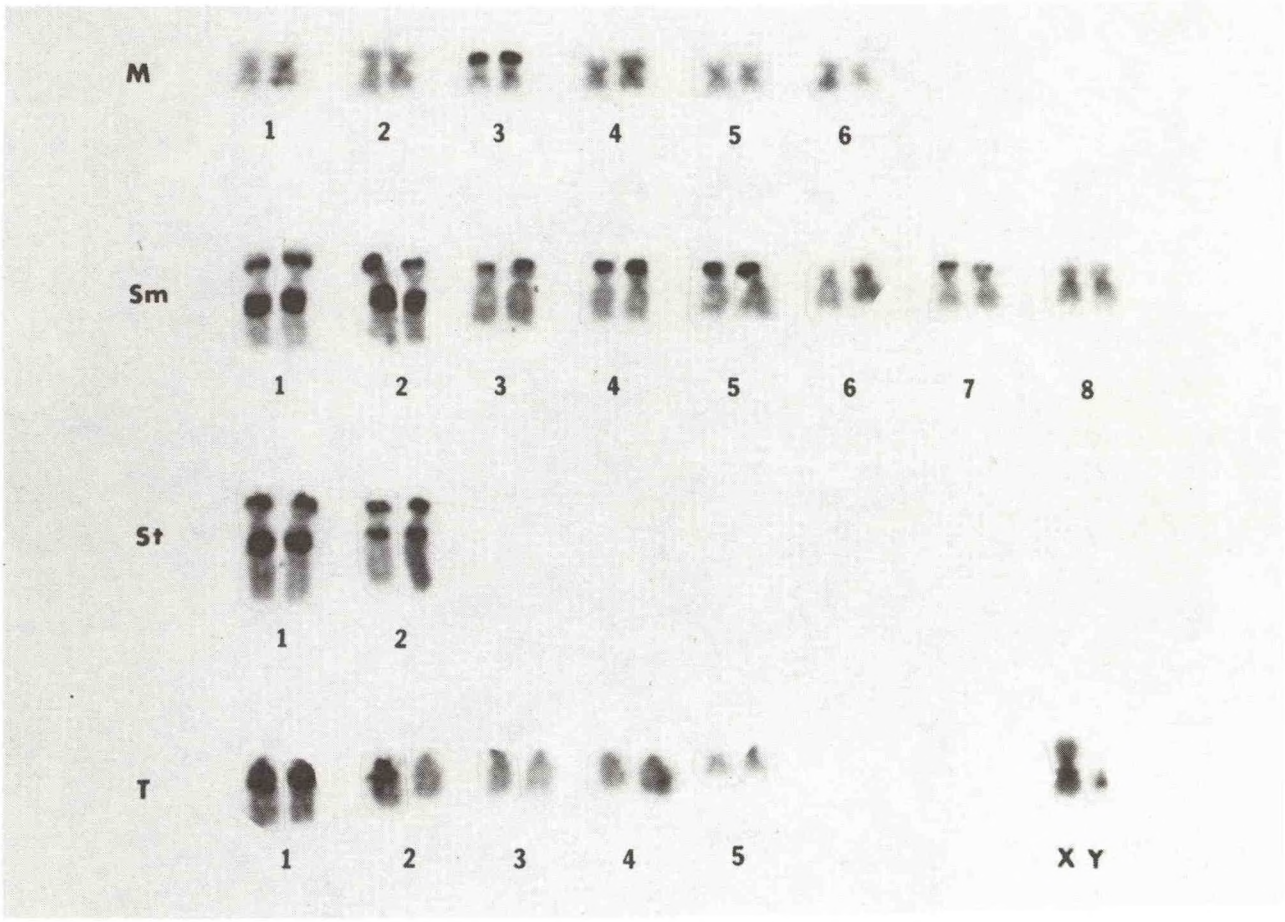


Figure 15. C-banded karyotype of dolphin 13 (male Stenella attenuata).

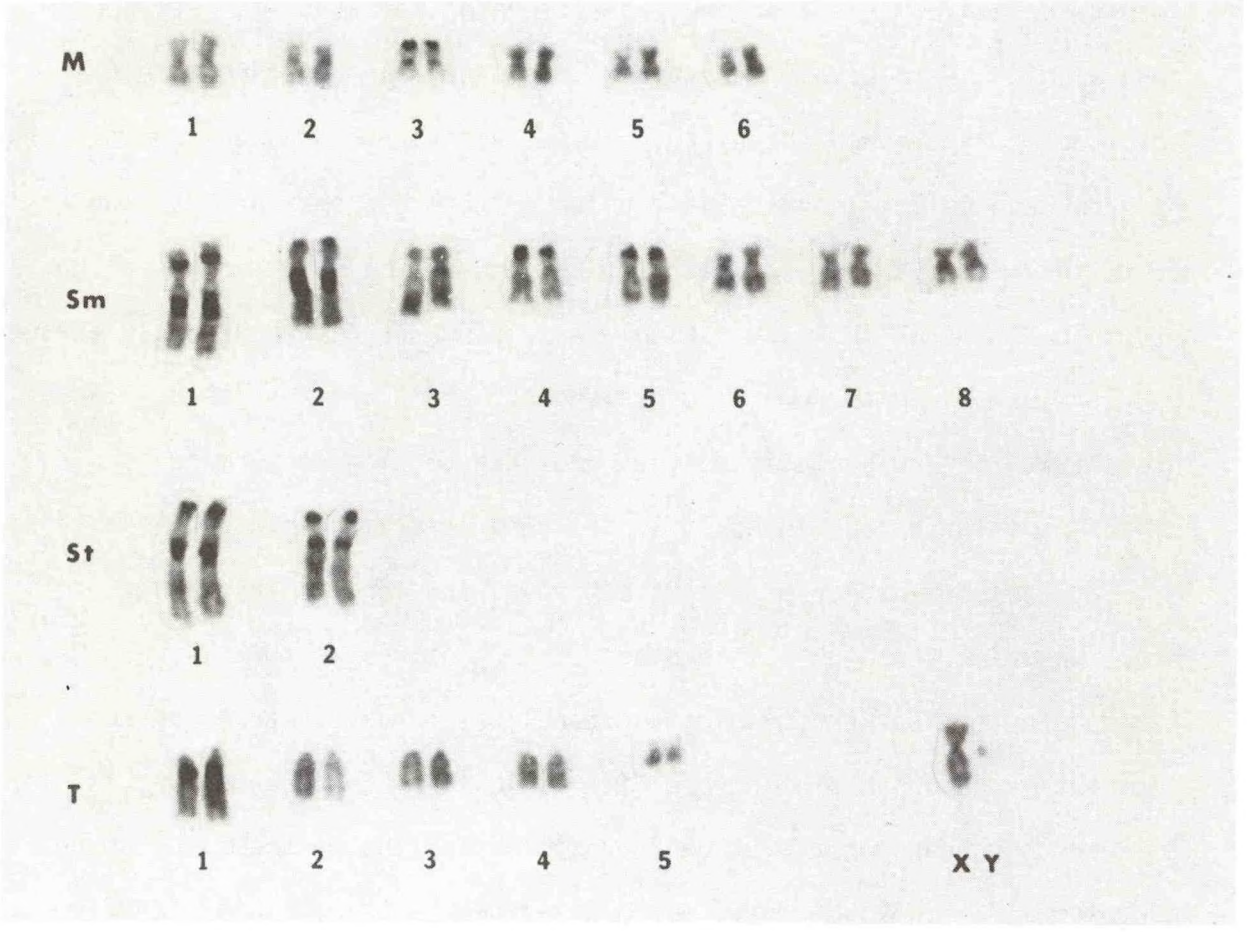


Figure 16. C-banded karyotype of dolphin 16 (male Stenella attenuata).

karyotypes for the four individuals of Stenella attenuata which were sampled. Figure 17 depicts a composite partial karyotype from the same four animals showing the major heterochromatin bearing pairs of chromosomes. This figure facilitates easy analysis of the known range of variation in heterochromatin found within the species. Variations in amount of heterochromatin (as indicated by extent and intensity of the C-positive stain) are common. In only one case in this species is a block of heterochromatin conspicuously absent in one homologue of a pair and present in the other. This situation occurs in SM2 in D1 in which the telomeric block of heterochromatin present in the p arm of one chromosome is conspicuously absent in the other. The interstitial heterochromatin on the homologue bearing no terminal heterochromatin is conspicuously large. The overall size of the block is as large as the combination of the interstitial and terminal blocks of heterochromatin found on its homologue. Chromosomes M1, M2, M4, M5, M6, SM6, SM8, and the two sex chromosomes in Stenella attenuata bear no detectable constitutive heterochromatin (see Figures 13-16). Chromosomes T2, T3, T4, and T5 frequently bear a trace of heterochromatin in the centromeric region. Chromosomes M3, SM3, SM4, SM5, and SM7 all bear a telomeric block of constitutive heterochromatin in the p arm. The amount of heterochromatin found in SM3 and SM7 is much reduced over that found in the other three chromosomes. Chromosomes SM2, ST1, and ST2 all bear a similar telomeric block of heterochromatin on the p arm but also bear an interstitial block of heterochromatin on the q arm. Chromosome SM1 bears a similar interstitial block on the q arm and also an interstitial, rather than a telomeric, C-band on the p arm. Chromosome T1 possesses an interstitial block on the q arm only.

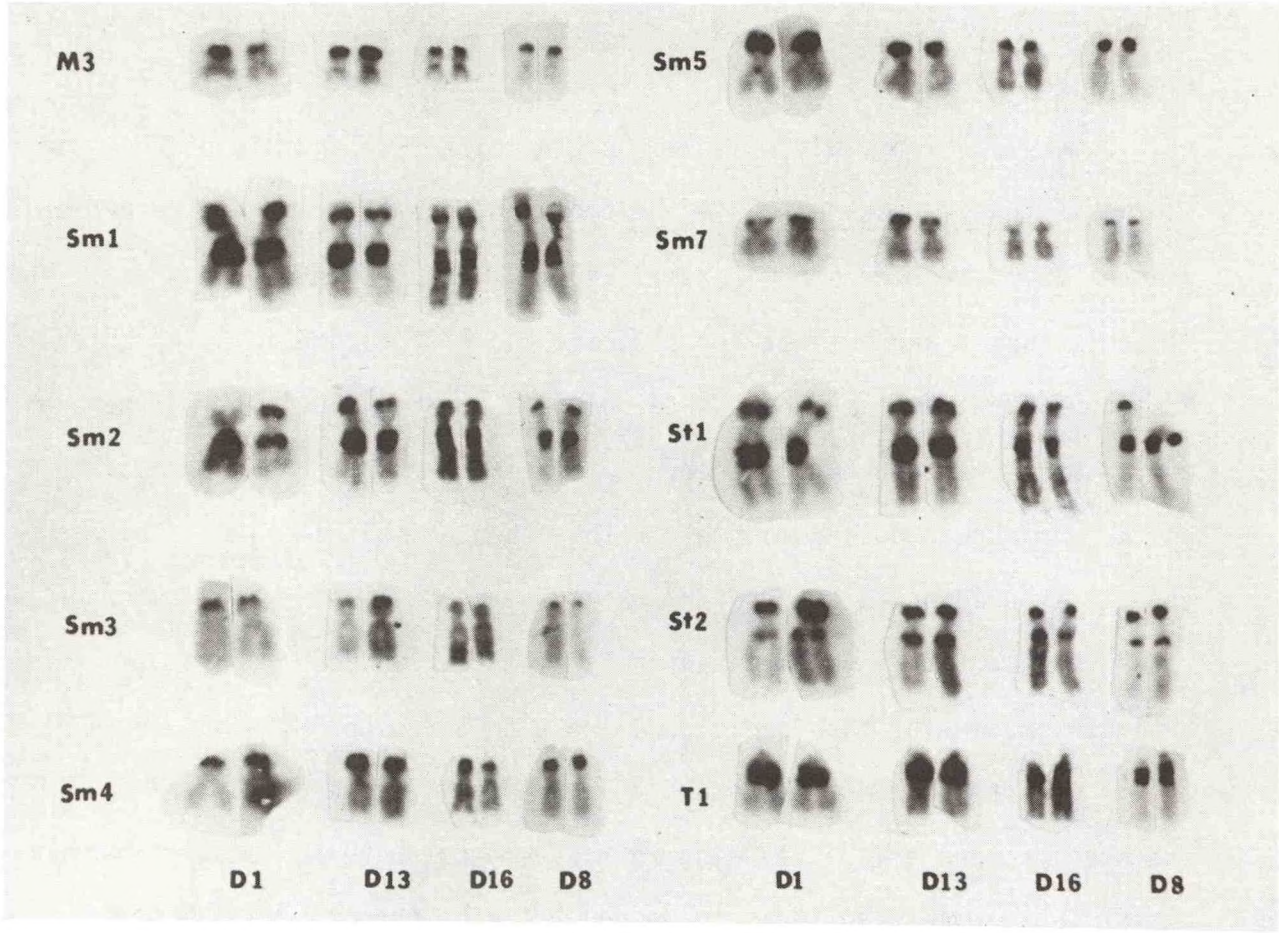


Figure 17. Comparison of the major heterochromatin bearing pairs of chromosomes from four spotted dolphins (Stenella attenuata).

It is noteworthy that most of the constitutive heterochromatin is present mainly as telomeric or interstitial blocks with no heterochromatin occurring near the centromere of most chromosomes. Only the acrocentric (telocentric) chromosomes T2, T3, T4, and T5 seem to possess any heterochromatin at the centromere and this is at very low levels.

Some variability was observed between different animals of the same species (Stenella attenuata) in the amount of constitutive heterochromatin present on particular chromosomes. Figure 17 demonstrates these differences. D13 possesses a larger, heavier staining block of telomeric heterochromatin on M3 and SM4 than do the other three dolphins of this species. D16 generally possesses a lesser amount of heterochromatin on each of its chromosomes than on the corresponding chromosomes of the other animals sampled. This is especially evident on SM3 and SM7 where the normal telomeric heterochromatin found in the other animals is entirely absent. Reduced amounts of heterochromatin for this individual are also noted on SM4, SM5, and T1. The amount of heterochromatin present on SM3 and SM4 seems quite variable within the species, and animals tending to possess more heterochromatin on members of one of those pair also seems to possess more heterochromatin on members of the other. Other than those variations mentioned above and the obvious telomeric heterochromatin absent on SM2 in dolphin 1 (discussed above) the variation in the C-bands within the species are negligible.

The C-banded karyotype presented for Stenella longirostris (Figure 18) shows striking similarities to the karyotypes presented for Stenella attenuata, but does show some consistent differences. If we ignore the balanced translocation found in this animal and try to analyze the karyotype as it most likely would have been without the translocation, (which is discussed in detail in Section 6.2.4, below) the following observations can be

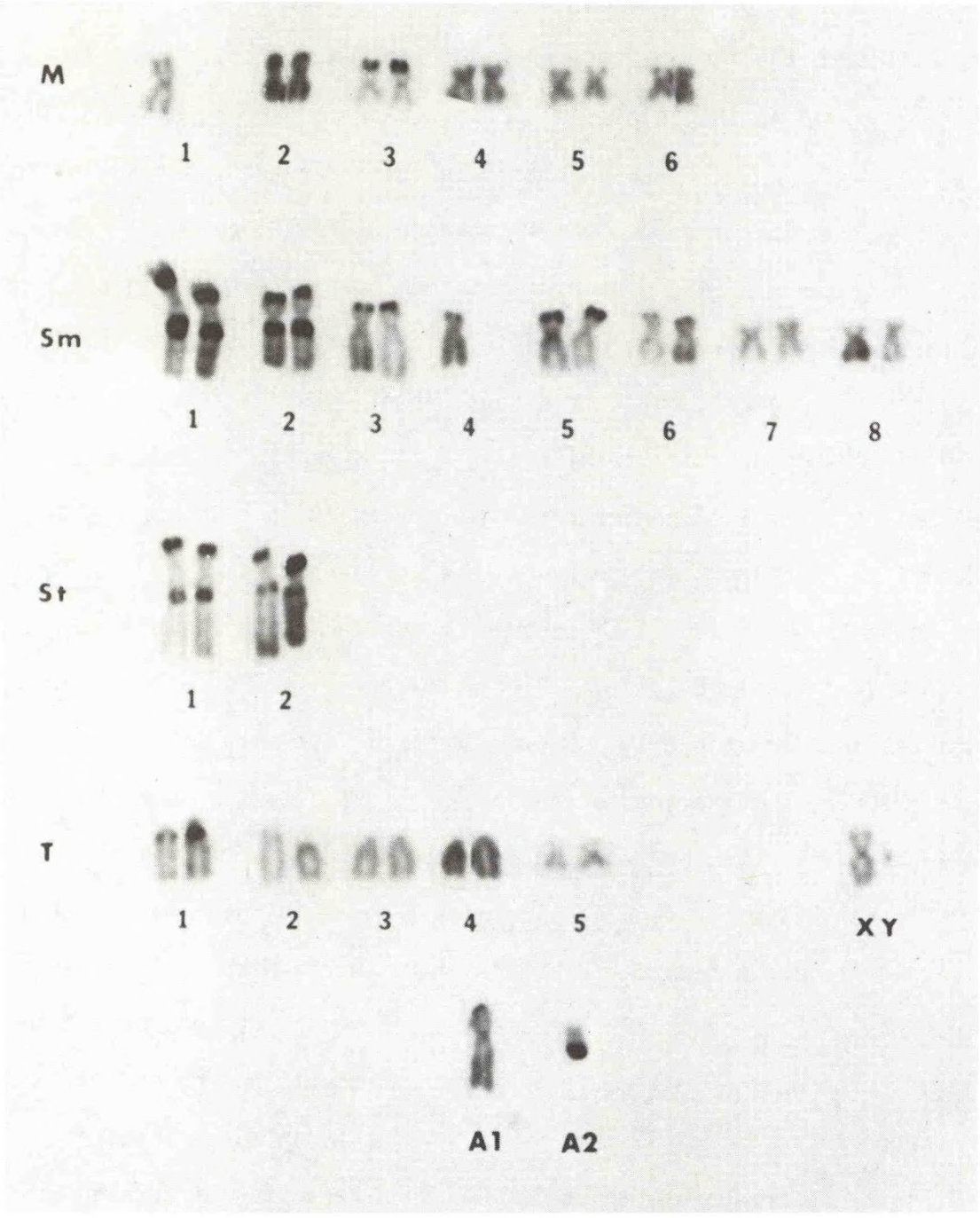


Figure 18. C-banded karyotype of dolphin 7 (male Stenella longirostris).

made. The existing chromosome SM4 and newly formed small chromosome which corresponds to the former p arm of the other SM4 are quite heteromorphic for heterochromatin in the telomeric block. The remaining chromosome SM4 shows a very slight amount of heterochromatin in that area and the translocated chromosome shows a large amount of heterochromatin in that area (see Section 6.2.4 for details). The amount of heterochromatin observed in chromosome T1 is greatly reduced over that found in S. attenuata, and chromosome SM7 possesses no telomeric block of heterochromatin on the p arm at all. Another major difference between Stenella longirostris and S. attenuata is the apparent total lack of centromeric heterochromatin on chromosomes T2, T3, T4 and T5 in S. longirostris. However, some preparations of S. attenuata also show no heterochromatin on these chromosomes; and with the present sample size, it is impossible to tell if this characteristic is also variable in S. longirostris. Additionally, chromosome SM6 in Stenella longirostris seems to possess a very small amount of telomeric heterochromatin in the p arm which is apparently lacking in Stenella attenuata.

The karyotype of Stenella plagiodon (Figure 19) bears a striking similarity to the karyotype for Stenella attenuata with the following exceptions. Chromosome SM7 possesses no telomeric heterochromatin on the p arm as is found in S. attenuata and the centromeric heterochromatin found on the acrocentrics T3 and T5 appears to be in heavier concentration.

Figure 20 presents a composite C-banded karyotype of selected chromosomes from a diploid cell (with one chromosome from each pair represented) for each of the three species discussed above. The heterochromatin content for corresponding chromosomes in each of the three species is generally quite consistant; however some consistant differences can be noted from this figure. The overall heterochromatin content of most of the chromosomes is less in

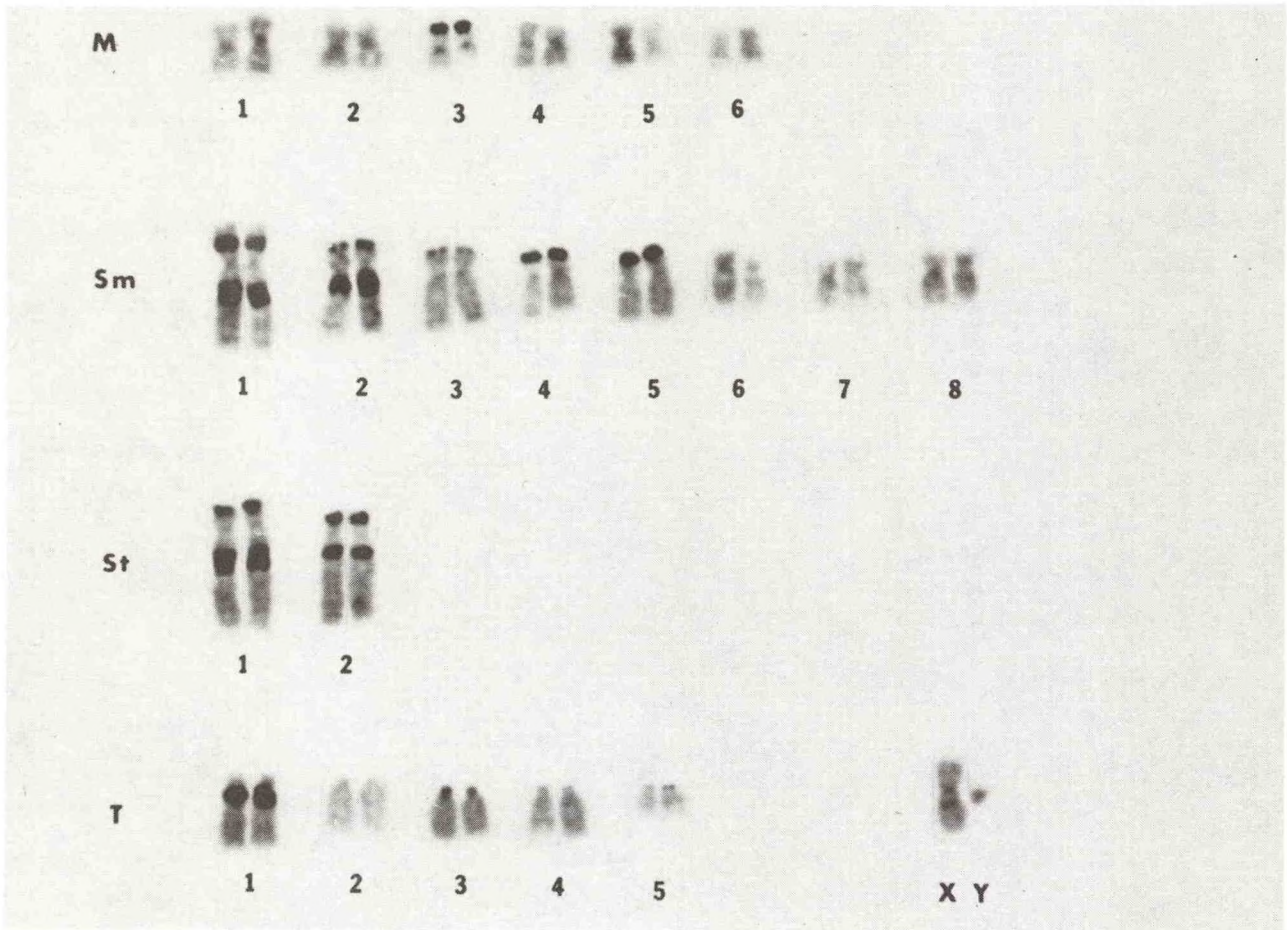


Figure 19. C-banded karyotype of a male Stenella plagiodon.

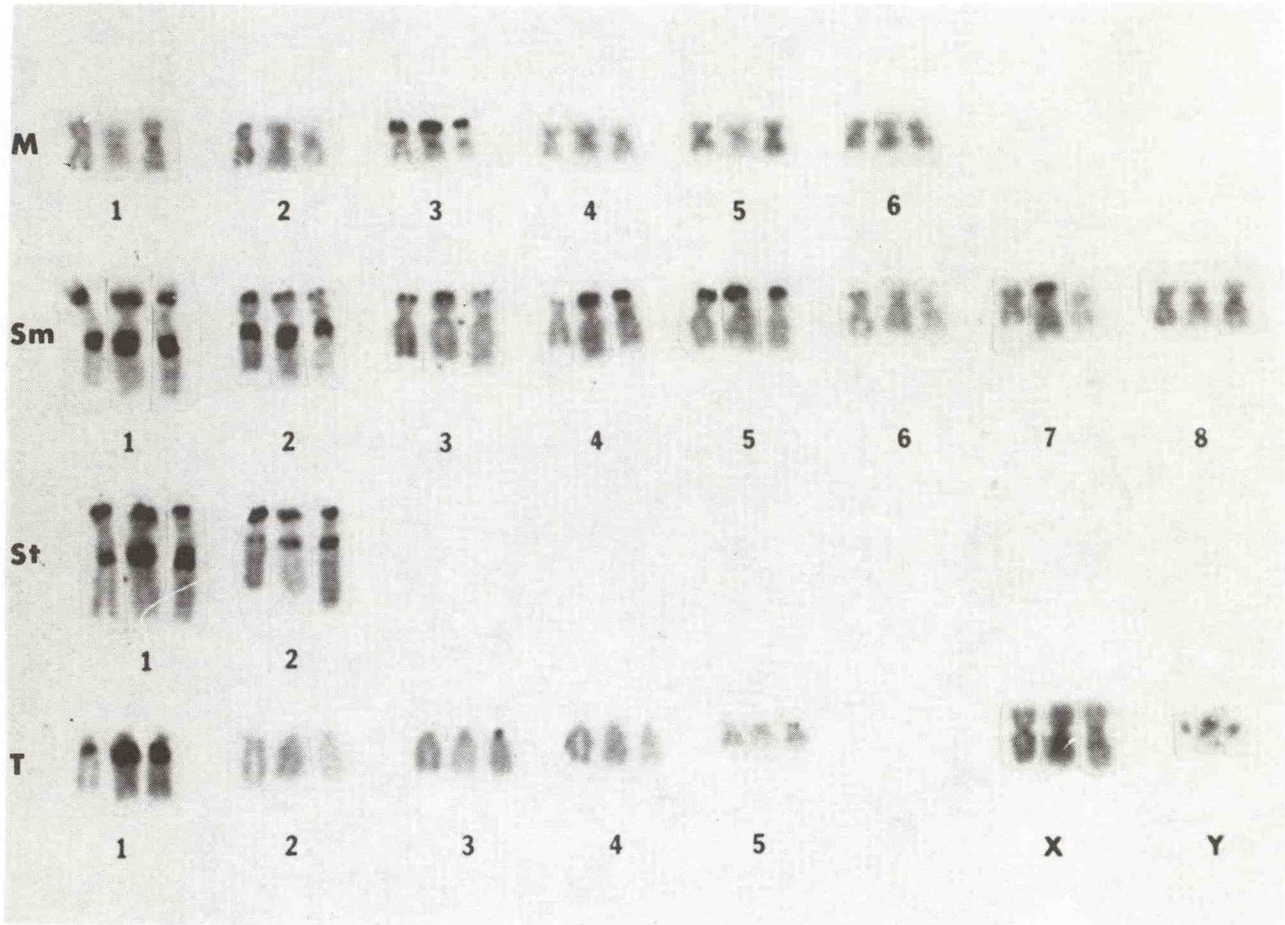


Figure 20. C-banded comparison of selected chromosomes (one from each pair) from diploid cells of three species of Stenella: (from left to right) S. longirostris, S. attenuata, and S. plagiodon.

S. longirostris and heavier in S. attenuata and S. plagiodon. Some obvious exceptions exist: SM2 for example has conspicuously more heterochromatin present in the telomeric block for S. longirostris than for either of the other two species. The conspicuous difference shown between the chromosomes of SM4 may be more pronounced in the example than in reality inasmuch as the portion of chromosome SM4 which broke away from the homologue not shown (SM4p) has conspicuously more heterochromatin than does the normal SM4 which is shown. The most conspicuous and consistent difference found between any of the species studied is the presence of the telomeric block of heterochromatin on SM7 in S. attenuata which is lacking in S. longirostris and S. plagiodon. Furthermore, a very small telomeric block of heterochromatin appears in SM6 of S. longirostris with no corresponding heterochromatin in either S. attenuata or S. plagiodon. A very small amount of telomeric heterochromatin is also found on the acrocentric chromosomes T3 and T5 in S. plagiodon but does not show on their counterparts in S. longirostris or S. attenuata.

6.2.3 Nucleolar Organizer Regions

Reasonably similar patterns of silver staining for nucleolar organizer regions were found in all individuals and all species examined. Some variations were noted, which remained consistent for a particular individual, but may not hold for the entire species. The only chromosomes which are involved in silver staining and thus possess nucleolar organizer regions are the telomeric chromosomes. The intensity of the silver staining regions differ between individual cell spreads within the same animal but the number of nucleolar organizer regions showing any activity seem to remain constant for a given animal. The locations of NOR activities also seem to remain constant for a given animal. D1 and D13 both possess three active NORs in the

telomeric chromosomes (see Figure 21). D1 has two active nucleolar organizer regions on chromosome T3 and one on one of the homologues of T5. The reverse situation is true in D13 where both homologues of T5 and only one homologue of T3 are involved. D8 and D16 show four active NORs, one on each of the homologues of chromosome T3 and T5.

The silver stained karyotypes of Stenella longirostris and Stenella plagiodon are both quite similar, each having a heavily staining nucleolar organizer region on each of the homologues of T3 and T5. Associations between homologues or non-homologues were commonly observed in all three species. Figure 22 shows four such associations for Stenella attenuata and S. plagiodon in various combinations. Such associations were commonly observed for all three species and seemed to have no specific differences or differences within a particular individual.

6.2.4 Balanced Translocation Found in Dolphin 7

Standard G-banded and C-banded karyotypes are presented for D7 (a phenotypically normal Stenella longirostris) in Figures 10 and 18, respectively. Both karyotypes clearly show a balanced translocation that exists between chromosome M1 and SM4. A detail of only the affected chromosomes and their homologues appear in Figure 23. Apparently, a break occurred in the q arm of SM4 slightly below the centromere, and the resulting chromosome piece of the q arm of SM4 reattached to the distal end of M1p. The resulting chromosome, if analyzed by gross karyotypic methods without banding, would appear quite similar to chromosome SM2 in terms of arm ratio, although it is slightly larger. Even with G-banding, there are some gross similarities between chromosomes SM2 and the translocated chromosome M1-SM4q. These include a darkly staining G-positive centromeric region, a light G-negative

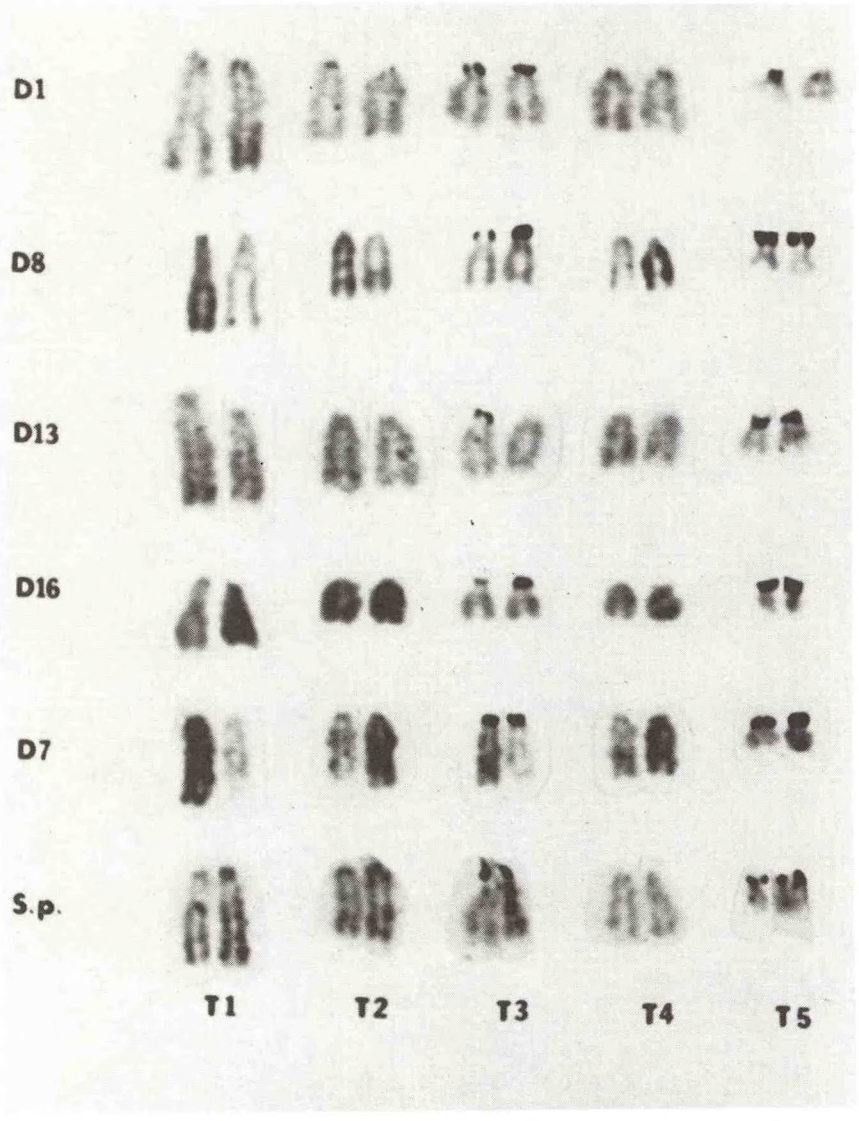
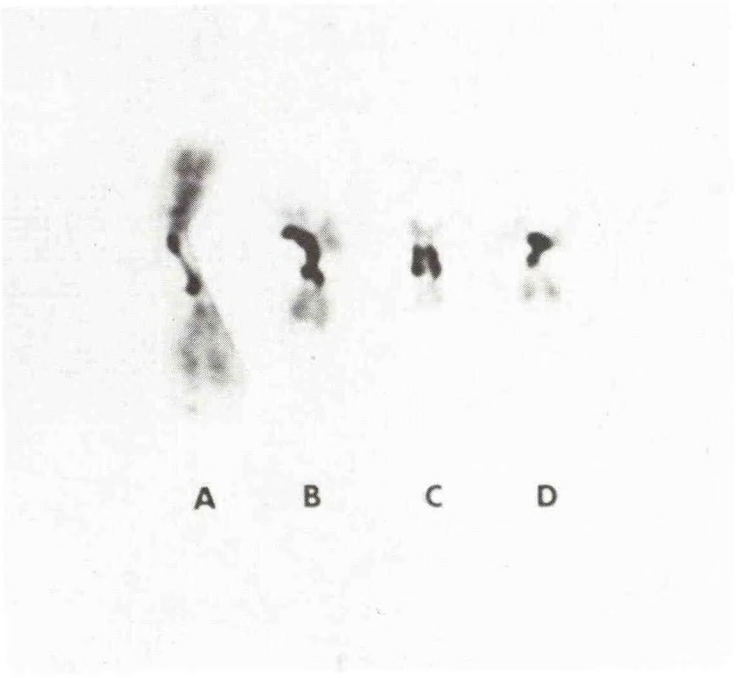


Figure 21. Partial silver stained karyotypes of the telocentric chromosomes showing distribution and activity of NORs for four individuals of Stenella attenuata (D1, D8, D13, and D16), a Stenella longirostris (D7), and a Stenella plagiodon (S.p.).



LEGEND:

A. Stenella plagiodon:
Association between T3-T3.

B. Stenella attenuata (D16):
Association between T2-T5-T5.

C. Stenella attenuata (D8):
Association between T5-T5.

D. Stenella attenuata (D8):
Association between T3-T5-T5.

Figure 22. Silver stained preparations showing associations commonly observed between various combinations of telocentric chromosomes.

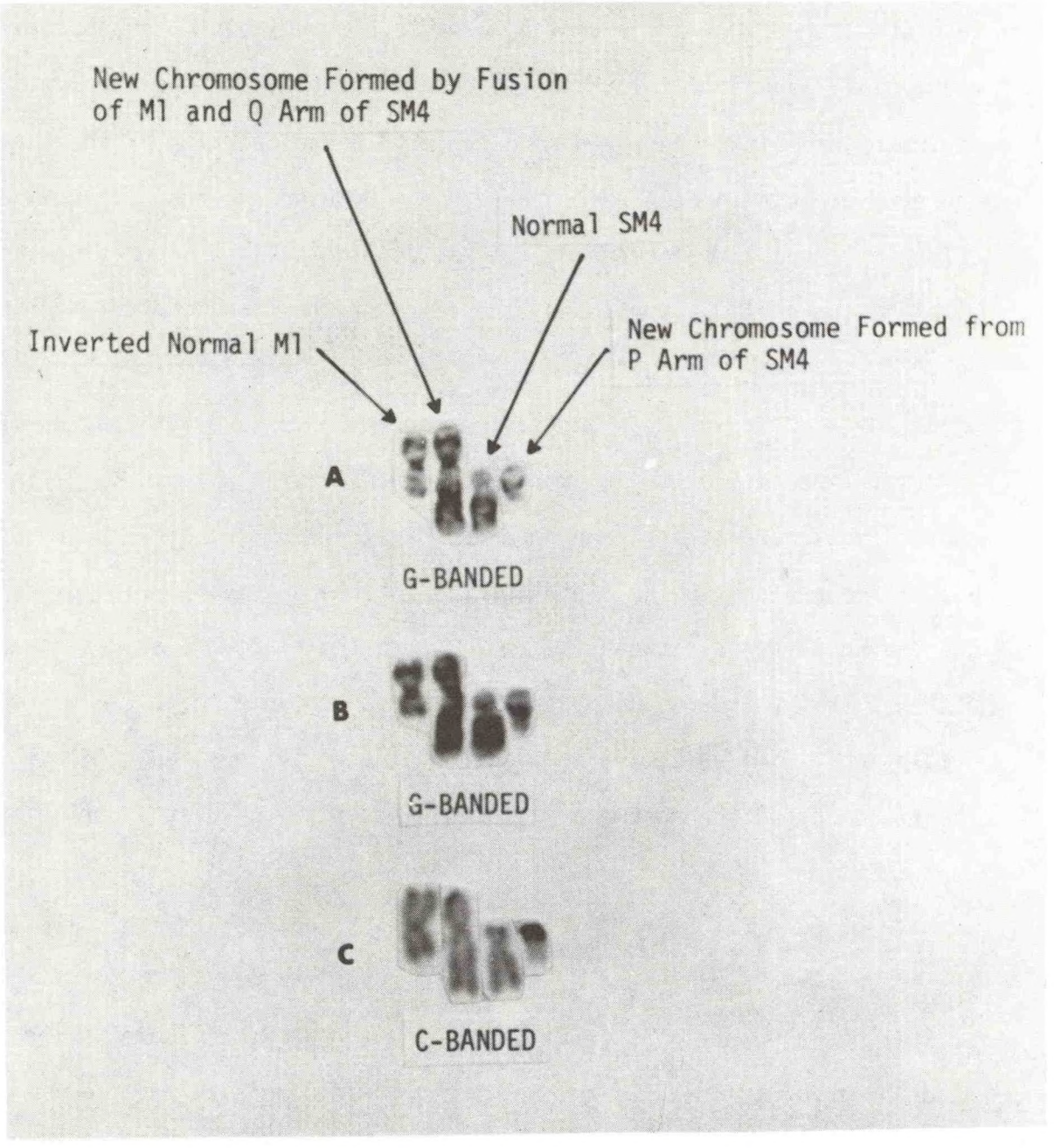


Figure 23. G- and C-banded chromosomes involved in the balanced translocation found in dolphin 7 (Stenella longirostris) and their normal counterparts.

telomeric portion of the p arm adjacent to a narrow but dark G-positive band, and a light G-negative block in the proximal portion of the q arm with a series of G-positive dark bands in the more distal portion of the q arm giving the entire distal area a dark appearance. However, several major important differences can be demonstrated. These include a large G-negative staining block in the proximal portion of the p arm of the translocated chromosome (only a very small G-negative band is found in the proximal portion of an SM2); and a series of much lighter, narrower G-positive staining bands in the distal portion of the q arm of the translocated chromosome, giving it a generally lighter staining appearance than the heavier, darker, broader G-positive regions in the distal portion of the q arm of SM2. Comparison of the G-band patterns between a normal M1 and the p arm and a proximal portion of the q arm of the resulting translocated chromosome reveals clear chromosome homologies. Similarly, the remainder of the distal portion of the q arm of the translocated chromosome bears homologies with the distal portion of a normal SM4.

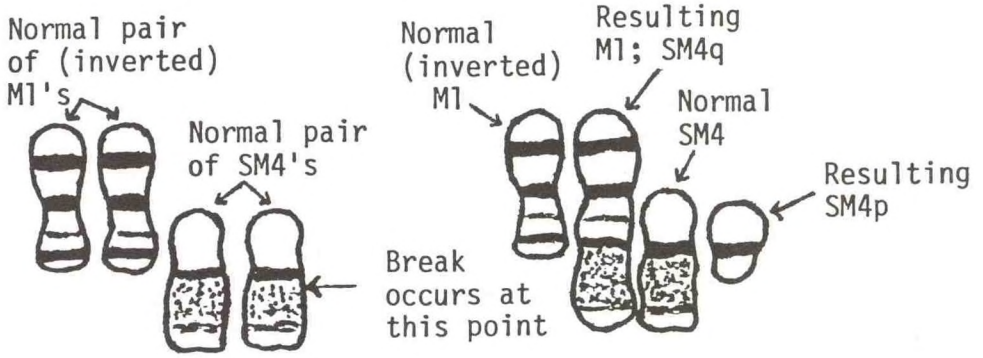
The remaining abnormal chromosome; which consists of the p arm of SM4, the centromeric region, and a small portion of the proximal end of the q arm of SM4; bears no striking resemblance to any other chromosome in the spread. No other chromosome exists in the spread of similar size and arm ratio. It is smaller than, and distinct from, M6 with which it could be confused in a gross karyotype by a careless observer. Even in gross karyology, however, the arm ratios are quite distinct inasmuch as M6 is a metacentric chromosome (which in some spreads may appear slightly submetacentric) and the resulting piece of translocated chromosome (SM4p) is clearly submetacentric in all preparations. Furthermore, it is much larger and possesses a drastically different arm ratio

than T5 which is the only other chromosome in the spread that even approximates it.

When analyzed with G-banding, the resulting SM4p bears virtually no similarity to anything else in the spread. The q arm of SM4p (which corresponds to the p arm of a normal SM4) shows a large telomeric G-negative block with a G-positive band adjacent to the centromere. The p arm appears to be entirely G-negative.

When analyzed using C-band comparisons, the identity of the translocated chromosome pair is equally obvious. As indicated above, the large translocated chromosome (M1-SM4q) is similar in size and arm ratio to SM2. However, it is very easily distinguished from SM2 by the conspicuous absence of the interstitial block of heterochromatin found on the proximal portion of the q arm of the latter chromosome. In some preparations, a small C-positive staining region was found as a telomeric block on the resulting M1-SM4q chromosome. The reason for this C-positive staining block is not clearly understood inasmuch as it corresponds to the terminal end of the q arm of a normal M1 which apparently bears no heterochromatin. The small SM4p has a large block of telomeric heterochromatin on the q arm which corresponds to the telomeric heterochromatin found on the p arm of SM4. The resulting amount of heterochromatin on the small translocated chromosome piece is much larger than that found on the remaining normal SM4, leading me to believe that the original homologues of SM4 prior to the translocation event were quite heteromorphic. Figure 23 shows two G-banded and one C-banded sets of the affected chromosomes aligned in such a manner as to facilitate easy identification. Figure 24 is a diagrammatic representation of the chromosomes showing the two pairs of homologous chromosomes prior to and following the translocation event.

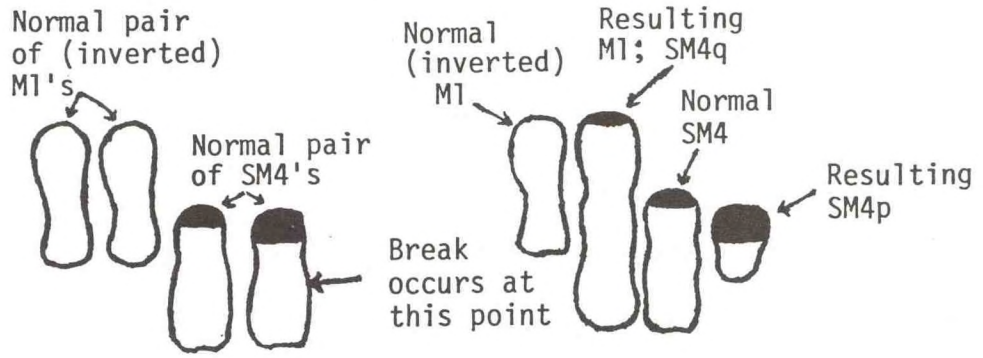
(a)



Normal Chromosome Compliment as Shown by G-Banding

Resulting Balanced Translocation 44t (M1; SM4q), SM4p as Shown by G-Banding

(b)



Normal Chromosome Compliment as Shown by C-Banding. (Note the heteromorphism in the amount of constitutive heterochromatin in the normal pair of SM4's).

Resulting Balanced Translocation 44t (M1; SM4q), SM4p as Shown by C-Banding. (Note the heteromorphism in the amount of constitutive heterochromatin in the normal SM4 and the resulting SM4p, and also the addition of heterochromatin to the newly formed M1; SM4q).

Figure 24. Diagramatic representation of the origin of the balanced translocation found in dolphin 7, Stenella longirostris, as observed by G- (24a) and C-banding (24b).

Inasmuch as harvests on this animal were made at an early passage and the translocation was observed in all cells examined, it is likely the translocation was present in the animal rather than just in tissue culture. With the present sample for the species (one animal), it is impossible to tell if the observed translocation is an aberrancy found only in the animal sampled or is more widespread in the population.

7.0 Discussion

One of the major stated objectives in the first year of this study was to gather and analyze dolphin tissue samples to determine the potential for use in chromosomal analysis in identifying stocks of dolphins in the eastern tropical Pacific (see Section 1.3). As a result of my opportunity to sail aboard the M/V Maria C.J., I was able to assess first hand the conditions aboard a working tuna seiner. I was also able to assess the feasibility of gathering tissue samples which were suitable for cytogenetic work from accidentally killed dolphins upon a working tuna seiner. I was not able to secure nearly as large of a sample size as I would have desired, but I was able to obtain tissue from a sufficient number of animals to test various collection procedures and cytogenetic protocols in our laboratory to determine which tissue types and growth procedures would be best for subsequent investigation.

The percentage of tissue returned to our laboratory which produced viable cultures was lower than we had hoped for, but the figures were in no way alarming inasmuch as one of the purposes in the first year of study was to determine which tissue types and storage protocols were the most effective. From the results of the first year it was obvious that lung and embryo are the two tissues of choice, and that McCoy's 5a (kept under refrigeration) is the storage medium which should be used on subsequent collections.

It is obvious that the more rapidly the tissues can be established, the greater the possibility of viability of those samples would be; however, it appears as if a large percentage of lung and embryo samples gathered in normal fishing operations would remain viable until received in our laboratory. This is especially true if we assume that the collection of tissue samples would

be somewhat equally distributed throughout the trip. Any of the samples collected during the later portion if the trip should retain a high viability.

The results of the tissue analyses in our laboratory indicate that we possess the capability of growing tissues which are collected on board working tuna seiners. We have shown that chromosomes from these tissue samples can be successfully banded in a manner which facilitates chromosome analyses.

Implied in the stated objective of analyzing initial samples for quality and for potential use of chromosomal banding in identifying stocks of dolphins in the eastern tropical Pacific, is the need for at least a preliminary discussion of the cytogenetics of the species and populations for which samples were obtained. The remainder of the discussion will be dedicated to the interpretation of our results in light of the work of others.

Karyotypic analysis has been a very fruitful method of elucidating relationships between populations within the same species. In fact in some rodent groups, karyotypic analyses have demonstrated that two populations thought to be only weak subspecies were indeed very distinct species totally incapable of interbreeding (Zimmerman and Lee, 1968). Other workers have also used karyotypic analyses to define full species where only subspecies were previously thought to exist (Hsu and Arrighi, 1968; Meier, 1968; Meier, et al., 1969; Soldatovic, et al., 1969; Malygin, 1970; Kozlovskii and Orlov, 1971; Wurster and Atkin, 1972; Kozlovskii, 1974; and Elder, 1978).

Data derived from comparisons of banding patterns in related species of bats (Stock, 1975), in related genera of primates (Stock and Hsu, 1973), lagomorphs (Stock, 1976), between muroid rodents belonging to different subfamilies (Mascarello, et al., 1974), and families of carnivores (Wurster-Hill and Gray, 1975) indicate that the organization of genetic

material as reflected in the G-band pattern is frequently very conservative. Barring extensive tandem fusions or numerous inversions, the G-band pattern can be recognized as more or less unchanged in related species, even between forms differing widely in karyotypic details. Such comparisons of G-band patterns between species and higher taxonomic categories are very useful in tracing phyletic relationships between taxa. When high diploid numbers are maintained (40+) in a given group, considerable G-banding pattern homology can be expected, whether one is comparing related species or members of related higher taxa. The degree of G-band homology generally decreases as more distantly related forms are compared, but it is important to note that each species (or population) tends to have its own general overall rate of G-band pattern rearrangement, which may be correlated with factors such as population size, life span, isolating mechanisms, and vagility. One would not expect carnivores or whales to change karyotypically as rapidly as small rodents due to relatively long generation intervals in the former groups. Thus far, G-banding studies on carnivores (Wurster-Hill and Gray, 1975), pinnipeds (Arnason, 1972), and cetaceans (Duffield, 1977; and Arnason, 1972, 1974 and 1980) support such a conclusion.

Arnason (1972) indicates that the karyotypic stability found in the Cetacea and Pinnipedia may be connected with the low reproductive rate (a factor of late sexual maturity and the fact that not more than one offspring is produced each year), good mobility, and the animals living in an environment without distinct niches. Some of these same factors could operate on other groups, such as carnivores.

Most previous work on the cytogenetics of various cetaceans (see, for example, Walen and Madin, 1965; Duffield, et al., 1967; Duffield Kulu, et. al., 1971; Arnason, 1969, 1970, 1972, 1974, 1980; Duffield, 1977; and Arnason,

et al., 1977), point to a high level of genetic conservatism in the Cetacea in general (including both the mysticete and odontocete groups). Virtually all species have a very similar karyotype in terms of number and gross chromosomal structure as reflected by either gross karyotype or G-band pattern homology. Three notable exceptions are the killer whale (Orcinus orca) (Duffield, 1977), the sperm whale (Physeter catodon) and the pygmy sperm whale (Kogia species) (Arnason and Benirschke, 1973). The general diploid chromosome component for most cetaceans is 22 pairs or 44 chromosomes, although several cetaceans (for example, Gervais' beaked whale (Mesoplodon europaeus) and Hubbs' beaked whale (M. carlhubbsi)) have a 2N number of 42 (Arnason, et al., 1977; Duffield, 1977). The sperm whale (Physeter catodon) (Atwood and Razavi, 1965; Arnason, 1970, and Arnason and Benirschke, 1973) and the pygmy sperm whale (Kogia breviceps) (Arnason and Benirschke, 1973) also show a 2N number of 42.

Those species of Stenella I studied have a diploid chromosome number of 44, which is in agreement with those published for delphinids by other workers such as Arnason (1974), Duffield (1977), and Arnason (1980). Arnason (1974, 1980) has demonstrated considerable chromosomal stability in delphinids when analyzed by gross karyotyping and G-band pattern analysis and has also shown a great deal of variability in the amount of constitutive heterochromatin. My findings are in agreement with those of Arnason (1974, 1980) inasmuch as this study has also demonstrated considerable G-band pattern stability coupled with variability of constitutive heterochromatin between species (discussed below).

I find an inconsistency in comparing the G-banded and C-banded karyotypes for Stenella plagiodon from this investigation with those of Arnason (published under the name of Stenella dubia by Arnason, 1974, and later called

Stenella plagiodon by Arnason, 1980). The G-band analyses in this paper and those presented by Arnason are identical but the C-band comparisons are in variance. Arnason shows M5 as bearing a telomeric block of heterochromatin in the p arm and M3 as being C-negative in that area. Careful analysis of the G-band patterns for the two chromosomes in question reveal that M5 has a G-positive band in the mid-region of the p arm which would not allow sufficient room for a telomeric block the size which Arnason shows. However, M3 has no G-positive staining area whatsoever in the p arm and appears to be the spot in which the heterochromatin would normally occur. Furthermore, the overall size of the M5 presented in the C-band karyotype in Arnason's paper appears to be somewhat larger than the M3 in the corresponding spread. These results are in variance with his list of absolute lengths given for ten cells of the animal being studied. These discrepancies lead me to the conclusion that in the C-banded karyotype in Arnason's paper, chromosome M5 is in reality a chromosome M3; although his arrangement in the G-banded karyotype is accurate.

I have considered the smallest metacentric chromosome (SM9 of Arnason, 1974 and 1980) as a telocentric chromosome and given it the number of T5. Arnason (1974) in a detailed study of the gross and banded karyotypes of Stenella dubia (= S. plagiodon), gave the mean arm ratio as 2.33 which falls well in his range for submetacentric chromosomes (R = 1.67 - 3.00). According to these measurements it is, in actuality, more closely associated with his metacentric chromosomes than with his subtelocentric chromosomes. However, analysis of the nucleolar organizer regions (see Section 6.2.3, and specifically Figures 21 and 22) indicates that the satellite bodies are telomeric in nature. Apparently Arnason was measuring the NOR or satellite stalks as opposed to an actual p arm on the chromosome in question. The

relationship that exists is further clarified as Duffield (1977) found short arms on some of the acrocentric chromosomes. She noted in the odontocetes that the variability in the short arms ". . . is most obvious with regard to the smallest chromosome pair which often exhibits distinct short arms of variable size despite its acrocentric-like characteristic of frequent involvement in satellite association." She also commonly found arm differences on the acrocentrics in mysticete karyotypes, but noted that in this group the differences were entirely heterochromatic.

The presence of the nucleolar organizer region on the telomeric position of chromosome T5 is unmistakable and warrants its inclusion as a telocentric (acrocentric) chromosome rather than as a submetacentric as has been listed by Arnason (1974). The present work, as a result of these findings, shows chromosome spreads arranged in this manner, and we would suggest that any subsequent works would also use this modification of the Arnason order.

Two aberrancies were noted in the karyotypes of the dolphins I examined. One of these involved the absence of the telomeric block of heterochromatin on chromosome SM2 in Dolphin 1. Analysis of the C and G-banded karyotype involving this chromosome leads me to believe that the reason for the heteromorphism between the two homologous SM2 chromosomes is the result of an inversion in one of the two homologues. The observed condition is consistent with this hypothesis. The absence of the telomeric block of C-positive heterochromatin in the C-banded karyotype and the absence of the G-negative block of telomeric heterochromatin in the G-banded karyotype coupled with the extra wide block of interstitial heterochromatin on the aberrant homologue, leads me to believe that an inversion has occurred. If a chromosome break occurred immediately above the interstitial block of heterochromatin and the broken off piece inverted and reattached, the resulting doubly wide inter-

stitial block of heterochromatin would thus be composed of the combination of the normal width interstitial block and the terminal block. This relationship would explain the reason for the excess width in the interstitial block of heterochromatin and would also explain the absence in the telomeric region. In such an inversion situation, the location of the centromere would be largely unaffected, inasmuch as the location of the break in the q arm of the chromosome would be roughly the same distance from the centromere as the normal terminal end of the p arm. Thus, following the inversion, the centromeric position would be largely unaltered. This inversion is noteworthy inasmuch as it is the only major variation in the G-banded karyotype found in any animal we examined of this species.

The other aberrancy was the balanced translocation found in Dolphin 7 (Stenella longirostris). As discussed in Section 6.2.4, this translocation is probably something which occurred in the individual sampled and not something which was caused by the vicissitudes of cell culture. To determine if indeed the karyotype were an aberrancy found in only the animal I sampled, I compared the karyotype I prepared with the karyotype of Stenella longirostris presented by Duffield (1977). Unfortunately, however, the latter karyotype is a gross karyotype making comparisons quite difficult. Comparisons were further obfuscated by the apparent misidentification of some of the chromosomes in the karyotype. For example, the first homologue shown for Duffield's chromosome C1 is apparently a misidentified X chromosome. The actual X chromosome was obviated by the relative arm ratio of that chromosome in comparison with the chromosome she refers to as the X. The actual X chromosome is more metacentric than the chromosome which she labels the X chromosome. Further interpretations on the Duffield karyotype were felt unwarranted. It is noteworthy that Duffield (1977) lists two possible balanced translocations

without evident phenotypic abnormality in the animals: one in Inia and one in Delphinapterus.

Recent development of techniques for visualizing nucleolar organizer regions (NORs) on chromosomes by the use of silver staining (Bloom and Goodpasture, 1976) has allowed accurate determination of NOR numbers and location in a variety of mammalian species. Elder (1978) demonstrated that closely related species of cotton rats, genus Sigmodon, possessed different numbers of NORs and that the structures occurred in different chromosomal regions characteristic for each species.

I commonly observed satellite associations between two pairs of telocentric chromosomes in many spreads. This is consistent with the results of Arnason (1974) who states "In most instances the homologues of the SM9 [= T5] pair were attached by their short arms, indicating that this arm might be involved in the formation of the nucleolus. . . . Two chromosomes of the t group were frequently seen associated to each other by their short arms." These chromosomes are probably the same two pairs in which I commonly observed associations. The presence of nucleolar organizer regions on the acrocentric chromosomes is apparently widespread within cetaceans. I observed NORs on members of two pairs of every species studied. Duffield, et al. (1978) found nonrandom association patterns (clustering of acrocentric chromosomes including direct satellite associations) in 70-80% of the cells they examined in the Atlantic bottlenose dolphin (Tursiops truncatus). Furthermore, Arnason (1974 and 1980) commonly observed satellite associations between what he referred to as the smallest pair of autosomes in most every species of cetacean which he investigated. In most cases it would appear that these small "submetacentric" chromosomes are homologous to T5 and therefore show a pattern common throughout the entire order. In many of the species studied by

Arnason, for example (Balaenoptera borealis, Balaenoptera acutorostrata and Delphinus delphis), associations were also observed between another pair of telocentric chromosomes. Duffield, et al. (1978) found satellites on all five pair of acrocentric chromosomes in Tursiops truncatus, although associations between more than two or three pairs of chromosomes within the same spread were rare. Delphinus delphis was somewhat unique inasmuch as Arnason (1974) made no mention of any metacentric chromosomes showing associations. It would appear that one or two pairs of telocentric chromosomes (including Arnason's smallest submetacentric as the smallest telocentric) are involved in some type of satellite association in most species.

The intensity of the stain on nuclear organizer regions is generally thought to indicate the activity level of that NOR (Miller, et al., 1976; Warburton, et al., 1976; Hayata, et al., 1977; Lau and Arrighi, 1977; Miller, et al., 1977; Schmid, et al., 1977; and Hansson, 1979). However, recent work (Kasprzak and Wyandt, 1979) indicate that the amount of time that harvested cells are allowed to remain in the glacial acetic acid - methanol fix can cause individual cells to show the same range and type of variability in staining as do NOR activity levels. Although I do not feel that this is a factor in the present study, a possibility does exist that the results may be affected in some way by this phenomenon.

In many groups of mammals one of the most active mechanisms giving rise to karyotypic variations involves addition, deletion, and other changes involving the constitutive heterochromatin (C-band regions) of chromosomes. In some groups of mammals, C-band variations may be the only chromosomal difference found between related species as described in bats by Stock (1975) and in rodents by Pathak, et al. (1973). Such mechanisms are certainly the most important in the cetaceans (Arnason, 1974 and 1980) where identical or

nearly identical G-banding patterns are found in most species, but the C-bands or constitutive heterochromatin varies greatly. Examples can be seen in other groups: in bats (Stock, 1975) and rodents (Arrighi, et al., 1974). Such variation in C-band material has even been used in distinguishing between subspecies of rodents (Arrighi, et al., 1974; Mascarello and Warner, 1974) and family groups in man (Pearson, et al., 1972).

In the three species of Stenella which I studied (Stenella longirostris, S. attenuata, S. plagiodon) all the positive staining regions of constitutive heterochromatin correspond to G-negative regions (light staining bands) on each of the chromosomes. However, I observed many additional G-negative regions which did not correspond to C-positive staining regions, and were thus not composed of heterochromatin. These results are consistent with those listed by Arnason (1974, 1980). I observed considerable heteromorphism in both the amount and location of constitutive heterochromatin between homologous pairs of chromosomes in the animals I studied. Variation in heterochromatin was also observed when comparing homologous chromosomes within one species (see Figure 17 in Section 6.2.2).

Both Duffield (1977) and Arnason (1974 and 1980) noted heteromorphism with respect to interstitial and terminal heterochromatin in virtually all the cetaceans they studied. In species such as Delphinapterus leucas and Balaenoptera physalus in which the interstitial and terminal blocks of heterochromatin are especially large, Duffield (1977) found the heteromorphic variation to be considerable. The presence of extensive polymorphisms in C-band heterochromatin is not unusual. It is found in high frequency in human populations (Craig-Holmes and Shaw, 1971; Craig-Holmes et al., 1973) and has also been well documented in primates (Ma and Jones, 1975), the Carnivora (Fredga and Mandahl, 1973) and the Rodentia (Forejt, 1973). Despite this

heteromorphism within individuals and within a species, consistent differences can be observed between the species which I investigated (see Figure 17 in Section 6.2.2) as well as the work of Arnason (1974, 1980).

In his analysis of the karyotype of Stenella clymene (Arnason, 1980) the C-band karyotype shows a telomeric block of heterochromatin on M3 as I have found it in all the species of Stenella I have investigated. It is noteworthy, however, that in Stenella clymene Arnason found telomeric heterochromatin present in SM6 and 8 and absent in 7: the exact opposite of the situation in S. attenuata. S. plagiodon has no heterochromatin in either SM6, 7, or 8 and S. longirostris possesses only a small amount of telomeric heterochromatin in SM6, and none in 7 and 8. Thus, the observed situation in Stenella clymene appears to be somewhat intermediate between the species that I investigated.

The function of heterochromatin is not clearly understood, and has served as a source of controversy since its discovery more than 40 years ago. Consensus indicates that constitutive heterochromatin (C-band positive preparations) consists of chromosomal localization of condensed and highly repetitive DNA sequences that have been "added to" to the genome (Yunis and Yasmineh, 1971; Arrighi and Saunders, 1973; Pathak, et al., 1973). Since such condensed heterochromatin does not transcribe in vivo, it can vary greatly between populations without being selected against or altering the ability of populations to interbreed.

Part of the reason for the controversy is undoubtedly the fact that more than one kind of heterochromatin exists: for example, Ganner and Evans (1971) list five different states of heterochromatin and Jalal, et al., (1974) list a total of 11 types. Miklos, et al., (1980) give an excellent review of the evolution of knowledge and theories related to the function of

constitutive heterochromatin over the last 40 years. They point out that the main changes they have observed are changes only in semantics: "namely substituting satellite DNA for heterochromatin and heterochromatin for inert material." Swanson (1957) postulates that as a result of heterochromatin "alterations in form, size and number of chromosomes can be accomplished without sacrifice of euchromatic portions of the chromosomes." Schmid (1967) states that "constitutive heterochromatin in mammalian karyotypes is an expendable part of the genome . . . . its main and important significance is considered of evolutionary nature, facilitating the production of viable chromosomal rearrangements and thereby contributing to reproductive isolation in the process of speciation." Gropp (1969) indicates that regions of heterochromatin are prone to enhance growth structural variation and that such gross karyotypic variation creates a very powerful cytologic isolation mechanism for a new species or for a population underway in speciation. Yunis and Yasmineh (1971) indicate that the role of constitutive heterochromatin is structural in nature inasmuch as "It may attract homologous chromosomes for initial alignment during meiosis . . ." or "It may establish 'single barriers' that provide means for evolutionary diversity and speciation."

Arnason (1974) states that "The presence of heterochromatin in interstitial positions may be assumed to facilitate recombination, as the redundancy of the heterochromatin would predispose to early pairing of these segments and of the adjacent euchromatin. The pronounced C-band heteromorphism in the cetaceans indicates that structural rearrangements, unequal crossing-over, duplication and deletion may take place within the heterochromatic block but so far this hypothesis has not been supported by experimental studies."

Corneo (1976) states that "satellite DNAs located in the constitutive heterochromatin in the chromosomes may function as sterility barriers between diverging incipient species. . . ." and ". . .seem not to be adaptive and to be a means of speciation independent of phylogenetic evolution. . . ."

Arnason, et al. (1978) indicate that satellite DNA facilitates both chromosomal and genic polymorphisms and is thus of great importance in speciation. Euchromatin-heterochromatin overlapping between homologous chromosomes is common at meiotic pairing. The frequent size hetermorphism between homologous heterochromatic segments presumably accentuates this overlapping. Thus crossing-over would be excluded in regions of such euchromatin-heterochromatin overlapping. As the overlapping is rectified progressively in the chromosome arms, crossing-over rates are unaffected distant to the euchromatin-heterochromatin junctions. Thus, ". . . genes in the proximity of the junctions are collectively inherited and selected, whereas genes distant to the heterochromatin will be independently assorted and selected."

Many other workers (Hatch, et al., 1976; Hatch & Mazrimas, 1977, Bush, et al., 1977; Pathak & Wurster-Hill, 1977; and Fry & Salser, 1977) all indicate that large amounts of satellite DNA are important in facilitating chromosome rearrangements and therefore, cytogenetic evolution. Pathak and Wurster-Hill (1977) indicate that "with a low amount of constitutive heterochromatin, however, it is conceivable that karyotypic changes, such as Robertsonian fusions, may be more difficult to achieve. This might partially account for the remarkable stability seen in the chromosome complement of the Felidae, as well as the extensive interfamilial G-banding conservatism seen within the Carnivora." In contrast to what is observed in the Carnivora, cetaceans possess large amounts of heterochromatin and still show widespread G-banding

conservatism within the entire order (Arnason, 1974; Arnason, et al., 1978; Arnason 1980). The difference in the relationship between that observed in the Carnivora and that observed in the Cetacea seems paradoxical: if it is not a contradiction, it points to different mechanisms involved in the maintenance of conservative karyotypes within the groups.

Another proposed function of constitutive heterochromatin is that of causing pairing difficulties when differences are observed between homologous chromosomes, with such changes generating infertility barriers and hence, speciation (Fry and Salser, 1977). I have observed pronounced heteromorphism in the amount of heterochromatin found between homologous chromosomes in the species I studied. Other workers (Arnason, 1974; Arnason, et al., 1978; Arnason, 1980) have also observed considerable heteromorphism between homologous chromosomes in cetaceans. If such factors do, indeed, operate in cetaceans causing infertility barriers and speciation, heterochromatic differences between members of a population may lead to sympatric speciation in the manner described by Fry and Salser (1977). Insufficient data exist at this time to indicate if sympatric speciation in this manner is a possibility in the Cetacea, although an extensive study of the C-band variation within closely related species and populations of dolphins may shed light on the possibility.

From the studies listed above concerning the function of constitutive heterochromatin three major theories are evident: 1) heterochromatin and satellite DNA differences between homologues lead to meiotic pairing problems; 2) large amounts of heterochromatin contribute significantly to chromosome rearrangements; and 3) heterochromatin functions in genome restructuring and speciation (Miklos, et al., 1980). These authors, however,

speak strongly against the involvement of satellite DNA (heterochromatin) in: chromosome recognition events, rearrangement events, or speciation events.

The results of the present study (as well as the results of several other studies--Arnason, 1974; Duffield, 1977; and Arnason, 1980) indicate that large amounts of heterochromatin and considerable heteromorphism in the amount of constitutive heterochromatin between homologues of various chromosomes are coupled with exceedingly conservative G-band patterns in terms of number and homology. Therefore, I agree that the proposed functions of constitutive heterochromatin listed above appear unlikely.

Jalal, et al. (1974) found at least three classes of heterochromatin (as observed by fluorescence) in the fin whale (Balaenoptera physalus): Q-negative, Q-dull, and Q-bright. Most of the C-bands in the fin whale (which are interstitial or telomeric in nature) showed relatively dull fluorescence. Some of the pericentric heterochromatin showed negative fluorescence and one interstitial block found on one of the chromosomes displayed bright fluoresence. AT-rich DNA is known to show bright Q-fluorescence but GC-rich DNA shows dull or no fluorescence. Thus a rough correlation can be made between heavy satellite DNA and dull and negative fluorescence, and between light satellite DNA and bright fluorescence (Jalal, et al., 1974). It should be noted, the characteristics reported by Jalal, et al. (1974) on the bouyant densities of DNA for the fin whale (B. physalus) are strikingly similar to the pattern observed by Arnason, et al. (1976) for the minke whale (B. acutorostrata) and dissimilar from that of the fin whale. As the species identification is unquestionable in the latter work, it points to a possible species misidentification by Jalal, et al. (1974).

Arnason, et al. (1978) were able to demonstrate at least two DNA satellites with distinct buoyant densities in neutral CsCl of p = 1.702/1.703

and p = 1.710/1.711 in each of three species of balenopetrid whales (minke whale, Balaenoptera acutorostrata; sei whale, B. borealis; and the fin whale, B. physalus). In situ hybridization showed that in each of the three species the p = 1.702/1.703 satellite was located in the centromeric-paracentromeric C-bands whereas the p = 1.710/1.711 satellite was located in the telomeric C-bands. The data presented by these authors were interpreted to mean that "the quantitative evolution of the satellite DNA sequences preceded species divergence of the balenopterids and that the satellite sequences have remained relatively unaltered since the divergence took place." If buoyant density and in situ hybridization data were obtained on delphinids similar results may be obtained.

It is highly noteworthy that each species studied has consistent constitutive heterochromatin markers on various chromosomes that, given a larger sample size, would most probably facilitate the positive identification of each of the species by means of C-band karyotyping. I would hope that given a larger sample size this same C-band variation would hold in a consistent manner for the various populations under investigation. With the present sample size constraint of this study, it is impossible to determine whether the variation in constitutive heterochromatin between populations would be great enough to facilitate easy identification of those populations. However, the results I have obtained in comparison to the works of Arnason (1974 and 1980) are encouraging that such consistent variation is a strong possibility. In addition to the heterochromatic variation, there is also the possible existence of a balanced translocation in the eastern spinner dolphin (S. longirostris) population. With the present sample size (one animal) it is impossible to tell if the translocation is something which is found only in that animal, or if the characteristic is set in some part or all of the

population. If the characteristic were set in the population, it would serve as an excellent chromosome marker for positive identification of the individuals within that population.

I believe that further analyses of the heterochromatic variations and other chromosomal characteristics in dolphin populations may yield important new information relating to identification of intraspecific population differences in dolphins that may be of great value to management of dolphin populations. Only the second year of the proposed two year study will be able to determine if indeed this is a fact.

8.0 Proposed Field Sampling Protocols for Second Year of Study

As one of the task objectives for the first year of our investigation we were asked to determine if the potential for use of chromosomal banding in identifying stocks of dolphins in the eastern tropical Pacific was large and, if so, to "design an experiment and a field sampling protocol to define stocks using samples to be collected in the NMFS observer program" (see items 3 and 4 in Section 1.3). As we have mentioned earlier, an insufficient sample of animals was obtained the first year to completely determine the range of cytogenetic variation between two populations of the same species; however, from the sample we were able to obtain, and from a comparison with the existing literature, we believe that the method holds considerable promise in determining individual stocks of dolphins. Therefore, this section is an attempt to design a meaningful experiment by which to identify dolphin stocks and also to outline a proposed field sampling protocol to insure obtaining sufficient samples given the vagaries of tissue sample availability and collection by personnel in the NMFS observer program.

From the knowledge we have assimilated during the first year of the study, the following research design is assessed as the best system by which to define stock boundaries of dolphins in the eastern tropical Pacific.

8.1 Experimental Design

The first task for any subsequent study would be the training of the scientific technicians that participate in the SWR program conducted on board working commercial seiners. This training would enable the scientific technicians to gather sterile tissue samples that would be adequate for use in our laboratory in tissue culturing. Prior to joining the commercial vessels, the NMFS technicians would be briefly trained by us in techniques we have

determined to be the most effective. In order to facilitate this training, one of our staff members (G. L. Worthen) would come to the Southwest Fisheries Center in La Jolla and conduct a series of brief seminars with the NMFS technicians in which slides would be presented showing proper dissections along with an oral discussion and question and answer period. If fresh or frozen dolphins were available at the Southwest Fisheries Center, it would be advantageous for us to do an actual dissection of the animal in such a way that each of the technicians would see, first-hand, specifically how tissue samples would be removed from a recently killed dolphin. Lung and embryo are the tissues of choice to be collected during the second year, as we determined in the first year of our study (see Sections 4.2 and 5.2). Therefore, training would center around removal of lung and embryo tissue.

In addition to the seminars, each participating technician would receive a set of written protocols completely outlining the tissue removal and storage techniques to be used while on board the tuna seiner. This set of written protocols would be supplied as a portion of the kit that would be given to each participating NMFS technician. We would envision that a completed kit would consist of the following items:

1. Written protocols outlining the procedures and materials to be used in obtaining specific dolphin tissue samples for cytogenetic analyses, and written instructions for shipment of tissue samples.
2. An adequate supply of sterile 15 ml. tubes containing McCoy's 5A medium in which to place collected tissue samples.
3. Sufficient packing material in which to store tissues in the ship's onboard walk-in refrigerator and in which to ship tissues to our laboratory in Logan.

4. A set of dissection and sterilization equipment to use in the removal of tissue samples consisting of the following:
   a. Two pairs of curved 6-inch forceps.
   b. Two pairs of 6-inch stainless steel surgical scissors.
   c. Two standard dissecting scalpels with approximately 20 replaceable blades.
   d. One spray bottle.
   e. Three to five gallons of 95% ethanol.
   f. Five to ten sterile surgical masks.

Items 1, 2 and 3 would be supplied by our laboratory in Logan. We would ask that the NMFS Southwest Fisheries Center would supply item 4. The collected samples would be air shipped to us as soon as possible: either when the ship returned to its home base in San Diego or when the ship reached another port of call for any reason.

To adequately conduct this research, we will need tissue samples from large numbers of dolphins over a large geographic area. By large numbers we mean a total of approximately 100 to 200 animals selected from the appropriate stocks of appropriate species from suitable geographic areas.

Our experience on board a working tuna seiner leads us to believe that the captain has complete control over the area fished and what type of fishing is done by that seiner. Furthermore, the area in which a particular seiner is going to fish is unknown to anyone other than the captain (and other captains in his code group) prior to departure. It is, therefore, virtually impossible to meaningfully plan tissue collections on particular seiners with the idea in mind of obtaining tissue from specific species from specific geographic areas. It would be virtually impossible for any observer to know, while he was on board a working tuna seiner, what tissue samples were being

gathered or what areas were being fished by other observers. Coordination of efforts between observers or between the National Marine Fisheries Service and their observers would seem unrealistic.

With the known vagaries of tuna seiner fishing, it would appear that any tissue samples collected would be done in an almost serendipitous manner. With this in mind, the only collection stratagem which seems reasonable is one in which representative tissue samples are collected independently by each participating NMFS observer on each participating cruise. This is especially true inasmuch as we are predicting that between 25% and 30% of the tissue samples received in our laboratory would produce viable tissue cultures (see Table 3 in Section 4.2 and also Section 5.2). Thus, the screening of which tissue samples would be the most meaningful in determining stock boundaries and which would have only secondary priority would be made in our laboratory. We fully realize that under this design we would be establishing many more tissues than would be necessary if we were in an a priori manner able to outline geographic areas and species within those areas to be sampled. Decisions on which tissues for which cultures were to be established on a first priority basis in our laboratory would be made on the following items:

1. The condition of the tissue when received in our laboratory. Such factors as contamination level, type of contamination, visual appearance (whether the tissue sample appeared to be viable or necrotic) would be taken into consideration here.
2. Length of time between the date the tissue had been collected and the date received in our laboratory. Table 3 in Section 4.2 indicates that the interval between the time of collection and the time the tissue is established can be a factor in tissue viability. We would attempt to establish the most viable tissues or those of the highest

predicted viability based on temporal data on a first priority basis, inasmuch as temporal parameters would intermesh with the other constraints listed here.

3. The species of animal from which tissue was obtained. We would attempt to get as wide a species representation from the samples as would be practicable. An attempt would be made to culture tissue from a representative sample of every species for which tissue was obtained by us in our laboratory.
4. The stock, or population, within a species from which tissue samples were received. An attempt would be made to culture a representative sample from each stock for which tissue was received. In this regard we would request that copies of porpoise life history forms would accompany each shipment of tissue so we would have access to the observer's identification of each specimen.
5. Geographic areas from which samples were obtained. We would attempt to culture tissue from animals from as wide geographic distribution within a particular stock as would be practicable. In assessing which tissue samples to establish based on a stock-geographic-area decision making process, known distributions of stocks and species would be utilized. Sources including (but not limited to) Perrin, Coe and Zwiefel (1976), Perrin, Miller and Sloan (1976), and Perrin, et al. (1977), and Smith, (1979) would be heavily used.

As was stated earlier, we would intend to grow tissue from between 100 to 200 animals selected by the above means. We would anticipate that an absolute minimum of 10 animals representing any particular stock would be essential in defining stock boundaries although we would prefer a larger sample. We would attempt to obtain tissue samples from as wide a geographical area within the

range of the stock as is possible. We realize that we are dependent on the scientific observers for tissue samples and that the scientific observers are in turn dependent on the tuna seiner captains for accidentally killed dolphins. This uncertainty in terms of tissue availability is what leads us to outline a procedure by which numerous tissue samples are gathered at sea by each observer, and then the tissue samples screened as necessary in our laboratory.

As samples would be received in our laboratory, they would be screened for viability and the other parameters listed above. Those samples which passed our initial screening process (which we would envision would be the great majority of the samples) would be established in tissue culture by the procedures listed under Section 5.1, above. Harvesting and banding protocols would follow those outlined in Section 6.1, above. Chromosomal analysis would be conducted on the finished G- and C-banded karyotypes, but would heavily concentrate on careful analysis of the C-banded variation between the populations. The rationale for the use of C-banded karyotypes for discriminating between the populations is adequately covered in Section 6.2 and Section 7.0, above, and shall not be repeated here.

A final report should be prepared and submitted on the stock structures of dolphin populations in the eastern tropical Pacific. The report should include sections on background, methods, results, conclusions, recommendations for promulgation of stock units and boundaries, and recommendations for any appropriate further research.

9.0 Literature Cited

Arnason, U. 1969. The karyotype of the fin whale. Hereditas 62:273-284.

Arnason, U. 1970. Karyotypes of a male sperm whale (Physeter catodon L.) and a female sei whale (Balaenoptera borealist Less). Hereditas 64:291-293.

Arnason, U. 1972. The role of chromosomal rearrangement in mammalian speciation with special reference to Cetacea and Pinnipedia. Hereditas 70:113-118.

Arnason, U. 1974. Comparative chromosome studies in Cetacea. Hereditas 77:1-36.

Arnason, U. 1980. C- and G-banded karyotypes of three delphinids: Stenella clymene, Lagenorhynchus albirostris and Phocoena phocoena. Hereditas 92:179-187.

Arnason, U., and K. Benirschke. 1973. Karyotypes and idiograms of sperm and pygmy sperm whales. Hereditas 75:67-74.

Arnason, U., K. Benirschke, W. W. Nichols, and J. G. Mead. 1977. Banded karyotypes of three whales: Mesoplodon europaeus, M. carlhubbsi and Balaenoptera acutorostrata. Hereditas 87:189-200.

Arnason, U., R., Pero, and A. Lima-de-Faria. 1976. Analysis of cetacean DNA. I. Appearance of a heavy DNA component after banding in CsCl gradients. Hereditas 84:221-224.

Arnason, U., I. F. Purdom, and K. W. Jones. 1978. Conservation and chromosomal localization of DNA satellites in balenopterid whales. Chromosoma 66:141-159.

Arrighi, F. E., and G. F. Saunders. 1973. The relationship between repetitious DNA and constitutive heterochromatin with special reference to man. Symp. Medica-Hoechst 6:113-133 Stuttgart-New York: Schattauer.

Arrighi, F. E., A. D. Stock, and S. Pathak. 1974. Chromosomes of Peromyscus (Rodentia, Cricetidae) V. Evidence of pericentric inversions. Chromosomes Today 335-341.

Atwood, R. P., and L. Razavi. 1965. Chromosomes of the sperm whale. Nature 207:328-329.

Bloom, S. E., and C. Goodpasture. 1976. An improved technique for selective silver staining of nucleolar organizer regions in human chromosomes. Hum. Genetics 34:199-206.

Bull, J. J. 1978. Sex chromosome differentiation: An intermediate stage in a lizard. Can. J. Genet. 20:205-209.

Bush, G. L., S. M. Case, A. C. Wilson, and J. L. Patton. 1977. Rapid speciation and chromosomal evolution in mammals. Proc. Nat. Acad. Sci. (Wash.) 74:3942-3946.

Carr, D. H., R. P. Singh, I. R. Miller, and P. L. McGeer. 1966. The chromosome complement of the Pacific killer whale (Orcinus rectipinna). Mamm. Chromo. Newsl. 22:208.

Claflin, A. J., T. I. Malinin, and D. G. Hutson. 1978. Preparation of human monolayer cell cultures from frozen tissues, p. 379. In Tissue Culture Association 29th Annual Meeting Program and Abstracts, June 4-8, 1978.

Corneo, G. 1976. Do satellite DNAs function as sterility barriers in eukaryotes? Evol. Theory 1:261-265.

Craig-Holmes, A. P., F. B. Moore, and M. W. Shaw. 1973. Polymorphism of human C-band heterochromatin. I. Frequency of varients. Amer. Journ. Hum. Genet. 25:181-192.

Craig-Holmes, A. P., and M. W. Shaw. 1971. Polymorphism of human constitutive heterochromatin. Science 174:702-704.

Duffield, D. A. 1974. Fluorescent karyotype of the California gray whale. Nat. Marine Fish. Bull. 36(4):25-28.

Duffield, D. A. 1977. Phylokaryotypic evaluation of the cetacea. Ph.D. dissertation, University of California, Los Angeles. 154 pp.

Duffield, D. A., S. H. Ridgway, and R. S. Sparkes. 1967. Cytogenetic studies of two species of porpoise. Nature 213:189-190.

Duffield, D. A., M. A. Spence, and C. Paquin. 1978. Evaluation of metaphase association patterns for acrocentric chromosomes in the order cetacea. Cytologia 43:541-549.

Duffield Kulu, D. 1972. Evolution and cytogenetics, pp. 503-527. In S. H. Ridgway (Ed.), Mammals of the sea: Biology and medicine, Chapter 8. Thomas Springfield, Illinois.

Duffield Kulu, D., I. Veomett, and R. S. Sparkes. 1971. Cytogenetic comparison of four species of cetaceans. Journ. Mamm. 52:828-832.

Elder, F. F. B. 1978. Chromosomal evolution of the cotton rats, genus Sigmodon (Rodentia, Muridae). Ph.D. dissertation, University of Illinois, Urbana. 142 pp.

Flower, W. 1883. On the characters and divisions of the family Delphinidae. Proc. Zool. Soc. London 1883:466-513.

Forejt, J. 1973. Centromeric heterochromatin polymorphism in the house mouse. Chromosoma 43(2):187-201.

Fraser, F. C. 1966. Comments on the Delphinoidea, pp. 7-31. In K. S. Norris (Ed.), Whales, dolphins and porpoises. University of California Press, Los Angeles.

Fraser, F. C., and P. E. Purves. 1960. Hearing in cetaceans. Bull. Brit. Mus. Nat. Hist. London, Zool. 7(1):1-140.

Fredga, K., and N. Mandahl. 1973. Autosomal heterochromatin in some carnivores. Nobel Symposium 23:104-117.

Fry, K., and W. Salser. 1977. Nucleotide sequences of HS- satellite DNA from kangaroo rat Dipodomys ordii and characteristics of similar sequences in other rodents. Cell 12:1069-1084.

Ganner, E., and H. J. Evans. 1971. The relationship between patterns of DNA replication and of quinacrine fluorescence in the human chromosome complement. Chromosoma (Berl.) 35:326-341.

Gropp, A. 1969. Cytologic mechanisms of karyotype evolution in insectivores, pp. 247-266. In K. Benirschke (Ed.), Comparative mammalian cytogenetics. Springer, New York.

Hansson, A. 1979. Satellite association in human metaphases. A comparative study of normal individuals, patients with Down's syndrome, and their parents. Hereditas 90:59-83.

Hatch, F. T., A. J. Bodner, J. A. Mazrimas, and D. H. Moore, II. 1976. Satellite DNA and cytogenetic evolution; DNA quantity, satellite DNA and karyotypic variations in kangaroo rats (Genus Dipodomys). Chromosoma (Berl) 58:155-168.

Hatch, F. T., and J. A. Mazrimas. 1977. Satellite DNA and cytogenetic evolution. Molecular aspects and implications for man, pp. 395-414. In R. W. Sparkes, D. F. Comings and C. F. Fox (Eds.), Human molecular cytogenetics. Academic Press, New York.

Hayata, I., M. Oshimura, and A. A. Sandberg. 1977. N-band polymorphism of human acrocentric chromosomes and its relevance to satellite association. Hum. Genet. 36:55-61.

Horrall, J. F., B. K. Taylor, and K. M. Taylor. 1968. Karyotypes of the Pacific killer whale, Orcinus orca (Linnaeus 1758) and the Brazilian tapir, Tapirus terrestris (Gray 1843). Mamm. Chromo. Newsl. 9:244-245.

Hsu, T. C., and F. E. Arrighi. 1968. Chromosomes of Peromyscus (Rodentia, Cricetidae) 1. Evolutionary trends in 20 species. Cytogenetics 7:417-446.

Jalal, S. M., R. W. Clark, T. G. Hsu, and S. Pathak. 1974. Cytological differentiation of constitutive heterochromatin. Chromosoma 48:391-403.

Kasprzak, R. A., and H. E. Wyandt. 1979. Methods of simultaneously staining NOR, c-dots, t-dots and chromosomal cores with $AgNO_3$ and ammoniacal silver in mammalian chromosomes. Mamm. Chromo. Newsl. 20(3):81-83.

Kasuya, T. 1966. Karyotype of a sei whale. Sci. Rep. Whales Res. Inst. 20:83-88.

Kasuya, T. 1973. Systematic consideration of recent toothed whales based on the morphology of tympano-periotic bone. Sci. Rep. Whales Res. Inst. 25:1-103.

Kellogg, R. 1928. The history of whales--their adaptation to life in the water. Quart. Rev. Biol. 3:29-76, 174-208.

Kozlovskii, A. I. 1974. Karyological differentiation of North American subspecies of hoofed lemmings. Doklady Biol. Science 219:499-502.

Kozlovskii, A. I., and V. N. Orlov. 1971. Karyological evidence for species independence of Sorex isodon Turov (Soricidae, Insectivora). Zool. Zh. 50(7):1056-1062.

Lau, Y. F., and F. E. Arrighi. 1977. Comparative studies of N-banding and silver staining of NORs in human chromosomes. In the Proceedings of the Joint Seminar and Workshop of the III Latin American Congress of Genetics: Aspects of the Chromosome Organization and Function, Montevideo, Uruguay, February 2-5 and 6-12, 1977, pp. 49-55.

Ma, N. S. F., and T. C. Jones. 1975. Added heterochromatin segments in chromosomes of squirrel monkeys (Saimiri sciureus). Folia Primatol. 24:282-292.

Makino, S. 1948. The chromosomes of Dall's porpoise, Phocoenoides dalli (True), with remarks on the phylogenetic relation of the Cetacea. Chromosoma 3:220-231.

Malygin, V. M. 1970. The systematics of the subspecies of the common field mouse. Vestnik Moskovskogo Universiteta Seriya V Biologiya Pochvovedenie 25(5):89-91.

Mascarello, J. T., A. D. Stock, and S. Pathak. 1974. Conservatism in the arrangement of genetic material in rodents. J. Mammalogy 55:695-704.

Mascarello, J. T., and J. W. Warner. 1974. Chromosome variations in the plains woodrat: a pericentric inversion involving constitutive heterochromatin. Experientia 30:90-91.

Mead, J. G. 1972. On the anatomy of the external nasal passages and facial complex in the Delphinidae (Mammalia: Cetacea). Ph.D. dissertation, University of Chicago.

Meier, M. N. 1968. Complex taxonomic analysis of the species based on the study of common voles (Genus Microtus). Zool. Zh 47(6):850-859.

Meier, M. N., V. N. Orlov, and E. D. Skhool. 1969. Use of data on karyological, physiological and cytophysiological analyses for isolating a new species of rodent (Rodentia, Mammalia). Doklady Akad. Nauk. SSSR 188(6):1411-1414.

Miklos, G. L. G., D. A. Willcocks, and P. R. Baverstock. 1980. Restriction endonuclease and molecular analyses of three rat genomes with special reference to chromosome rearrangement and speciation problems. Chromosoma 76:339-363.

Miller, D. A., V. G. Dev, R. Tantravahi, and O. J. Miller. 1976. Suppression of human nucleolus organizer activity in mouse-human somatic hybrid cells. Exp. Cell Res. 101:235-243.

Miller, D. A., R. Tantravahi, V. G. Dev, and O. J. Miller, 1977. Frequency of satellite association of human chromosomes is correlated with amount of Ag-staining of the nucleolus organizer region. Am. J. Hum. Genet. 29:490-502.

Mitchell, E. D. (Ed.). 1975. Review of biology and fisheries for smaller cetaceans. Journ. Fisheries Res. Board, Canada 32(7):875-1240.

Nishiwaki, M. 1963. Taxonomical consideration on genera of Delphinidae. Sci. Rep. Whales Res. Inst. 17:93-104.

Nishiwaki, M. 1972. General biology, pp. 3-204. In S. H. Ridgway (Ed.), Mammals of the sea; biology and medicine, Chapter 1. Thomas, Springfield, Illinois.

Pathak, S., T. C. Hsu, and F. E. Arrighi. 1973. Chromosomes of Peromyscus (Rodentia: Cricetidae). IV. The role of heterochromatin in karyotypic evolution. Cytogent. Cell Genet. 12:315-326.

Pathak, S., and D. H. Wurster-Hill. 1977. Distribution of constitutive heterochromatin in carnivores. Cytogenet. Cell Genet. 18:245-254.

Pearson, P. L., J. P. M. Geraedts, and A. G. J. M. van der Linden. 1972. Human chromosome polymorphism, pp. 201-213. In Symposia Medica Hoechst 6: Modern aspects of cytogenetics: constitutive heterochromatin in man. Verlag, New York. 248 pp.

Perrin, W. F. 1977. Section on biological research, pp. 51-53. In F. Ralston (Ed.), A workshop to assess research related to the porpoise/tuna problem, Feb. 28 and March 1-2, NMFS, SWFC Admin. Rep. No. LJ-77-15. 119 pp.

Perrin, W. F., J. M. Coe, and J. R. Zweifel. 1976. Growth and reproduction of the spotted porpoise, Stenella attenuata, in the offshore eastern tropical Pacific. Fish. Bul. 74(2):229-269.

Perrin, W. F., D. B. Holts, and R. B. Miller. 1977. Growth and reproduction of the eastern spinner dolphin, a geographical form of Stenella longirostris in the eastern tropical Pacific. Fish. Bull. 75(4):1-26.

Perrin, W. F., R. B. Miller, and P. A. Sloan. 1976. Reproductive parameters of the offshore spotted dolphin, a geographical form of Stenella attenuata, in the eastern tropical Pacific, 1973-75. Nat. Marine Fish. Serv. Admin. Rep., LJ-76-34, 20 pp.

Prasad, N., D. M. Mumford, P. B. Barsales, T. Whithman, and J. R. Wilbur. 1970. Cytogenetic studies of dolphin (Tursiops truncatus) by an extended tissue culture technique. Experientia 26:1167-1168.

Rice, D. W. 1976. A list of the marine mammals of the world. NOAA Tech. Rep. NMFS-SSRF, in press.

Rice, D. W., and V. B. Scheffer. 1968. A list of the marine mammals of the world. U.S. Fish Wildl. Serv. Spec. Sci. Rep. Fish. 579:1-16.

Schenkkan, E. J. 1973. On the comparative anatomy and function of the nasal tract in odontocetes (Mammalia: Cetacea). Bijdragen Tot de Dierkunde 43(2):127-159.

Schmid, M., F. J. Hofgartner, M. T. Zenzes, and W. Engel. 1977. Evidence for postmeiotic expression of ribosomal RNA genes during male gametogenesis. Hum. Genet. 38:279-284.

Schmid, W. 1967. Heterochromatin in mammals. Arch. Julius Klaus Stift. Vererbungsforseh. 42:1-60.

Simpson, G. G. 1945. The principles of classification and a classification of mammals. Bull. Amer. Mus. Nat. Hist. 85:xvi + 350 p.

Slijper, E. J. 1936. Die Cetacean. Vergieichend-anatomisch und systematisch. Capita Zool. 7:1-590.

Smith, T. 1979. Report of the status of porpoise stocks workshop. Southwest Fisheries Center Administrative Report, No. LJ-79-41, 120 pp.

Soldatovic, B., B. Djulic, I. Savic, and D. Rimsa. 1969. Chromosomes of two species of the genus Apodemus (A. agrarius and A. mystacinus - Mammalia, Rodentia) from Yugoslavia. Arhiv. Biol. Sci. 21(1):27-32.

Stock, A. D. 1975. Chromosome banding pattern homology and its phylogenetic implications in the bat genera Carollia and Choeroniscus. Cytogenetics and Cell Genetics 14:34-41.

Stock, A. D. 1976. Chromosome banding pattern relationships of hares, rabbits and pikas (Order Lagomorpha): a phyletic interpretation. Cytogenet. Cell Genet. 17:78-88.

Stock, A. D., and T. C. Hsu. 1973. Evolutionary conservatism in arrangement of genetic material: a comparative analysis of chromosome banding between the Rhesus macaque (2n=42, 84 Arms) and the African green monkey (2n=60, 120 Arms). Chromosoma 43:211-224.

Sumner, A. T. 1972. A simple technique for demonstrating centromeric heterochromatin. Exp. Cell Res. 75:304-306.

Swanson, C. P. 1957. Cytology and cytogenetics. Prentice-Hall, New York.

Tomilin, E. G. 1957. Mammals of the USSR and adjacent countries. Vol. X (Cetacea). Israel Programme for Scientific Translation, Jerusalem.

True, F. W. 1889. Contributions to the natural history of the cetaceans, a review of the family Delphinidae. Bull. U.S. Nat. Hist. Mus. 36:192.

Walen, K. H., and S. H. Madin. 1965. Comparative chromosome analyses of the bottlenosed dolphin (Tursiops truncatus) and the pilot whale (Globicephala scammoni). Amer. Nat. 99:349-354.

Walker, E. P., F. Warnick, S. E. Hamlet, K. I. Lange, M. A. Davis, H. Uible, and P. F. Wright. 1975. Mammals of the world. Vol. 2, pp. 1083-1145. John Hopkins University Press, Baltimore.

Warburton, D., K. C. Atwood, and A. S. Henderson. 1976. Variation in the number of genes for rRNA among human acrocentric chromosomes: Correlation with frequency of satellite association. Cytogenet. Cell Genet. 17:221-230.

Winge, H. 1921. Review of the interrelationships of the Cetacea. Smithsonian Misc. Collections 72(8):1-97.

Worthen, G. L. 1980. An annotated computerized bibliography of the use of karyotypic analysis in the subspecific taxonomy of mammals. Unpublished report to NOAA, NMFS for Contract No. NASO-79-00205, 154 pp.

Wurster, D. H., and N. B. Atkin. 1972. Muntjac chromosomes: a new karyotype for Muntiacus Muntjak. Experientia 28(8):972-973.

Wurster-Hill, D. H., and C. W. Gray. 1975. The interrelationships of chromosome banding patterns in Procyonids, Viverrids and Felids. Cytogenet. Cell Genet. 15:306-331.

Yablokov, A. V., V. M. Bel'kovich, and V. I. Borisov. 1974. Whales and dolphins. Part II. Joint Publications Res. Ser., Virginia.

Yunis, J. J., and W. G. Yasmineh. 1971. Heterochromatin, satellite DNA, and cell function. Science 174:1200-1209.

Zimmerman, E. G., and M. R. Lee. 1968. Variation of chromosomes of the cotton rat, Sigmodon hispidus. Chromosoma 24:243-250.