A molecular analysis of the ostrich *Struthio camelus massaicus* communal nesting system.

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Thesis submitted in application for the degree of Doctor in Philosophy (Ph.D.) to the University of St. Andrews, 2000.

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ABSTRACT

Seven hypervariable molecular microsatellite markers were isolated and characterised to investigate the colonial nesting of the East African ostrich subspecies *Struthio camelus massaicus.* The study was based at Nairobi National Park, a 117 km^2 park just 7 km south of the city centre.

The ostrich breeding system is complex: I estimated that the territorial male defends a territory of 1.15 ± 0.27 (SD) km^2 when nesting. Females have larger breeding home ranges that overlap several male territories. From parentage analysis, I estimated that 3-7 females mate with a territorial male and lay up to 66 eggs in a communal nest within the male's territory. One female, the first (major female) to initiate egg laying in a nest, pairs with the territorial male and provides parental care in the form of egg guarding, incubation and escort of the chicks.

What makes the communal nesting system of the ostrich unique is that the major female gives free access to other (minor) females to lay in her nest. The major female, who lays a mean of 9.15 ± 2.47 (SD) eggs partitions the clutch into a central clutch comprising an average of 22.9 ± 3.7 (SD) eggs for incubation and peripheral clutch consisting of excess eggs that are not incubated. This study found that the major female may be able to select her eggs for retention in the central clutch. I investigated

the possibility that the other central clutch eggs retained were laid by her close relatives. This was not the case.

Both the territorial males and the major female had incubated extra pair fertilised eggs. This study found that 71.2% of the incubated eggs were not parented by either the territorial male or the major female. All the major females were found to lay in other nests as minor females, probably before becoming a major female.

The conflict arising from communal nesting and biparental care results in the territorial male copulating with his major female and other females venturing into his territory; the female also seeks extra pair matings and selectively favours her eggs for placement into the central clutch where they are incubated by herself and the territorial male. The major female and the territorial male both seek to maximise their individual reproductive success.

Chapter 1

GENERAL INTRODUCTION

1. The ostrich breeding system

1.1. Preamble

Ostriches are nidifugous species with a unique breeding system unlike any other birds. They have synchronised breeding and their annual season covers approximately five months from initiation to nest leaving. The breeding pattern is characterised by polygynandrous mating, with groups of females ranging over several males' territories and copulating with these males. Several females deposit eggs in a communal nest in the territories of these males. The females are made up of a major hen who initiates egg laying in the nest and contributes to parental investment, along with the territorial male, in the form of guarding, incubating, brooding and escorting the chicks (Sauer & Sauer 1966, Hurxthal 1979, Bertram 1992). Several minor female categories were recognised by Bertram (1992): pure minor females who exclusively laid in other females' nests, major females whose nests had been destroyed, future major females who laid as minor females before starting their own nests as major females or current major females who were laying as minor females although

they had an extant nest. This last category was found to be rare: out of 11 nests studied only 1 female fell into this category (Bertram 1992).

Up to 67 eggs may be deposited in a single nest while the nesting pair can incubate only 20-21 eggs. Eggs, in excess of the number that can be incubated, are ejected into a peripheral clutch that is not incubated and does not develop (Hurxthal 1979, Bertram 1992). The major female lays 7-11 eggs (Hurxthal 1979, Bertram 1992) and the other eggs are contributed by minor females, who are allowed, by the major female, to lay freely in the nest (Hurxthal 1979, Bertram 1992). There are no data showing why the female is constrained to lay an average of 10 eggs. Farm data however, indicate a reduction in hatchability in eggs stored past 10 days (Gonzalez *et al.*1999). Reduced hatchability and increased likelihood of predation may be some of the constraints limiting the number of eggs laid by the major female.

Females only mate with territorial males within the males' territories and the territorial male forms a pair bond with a major female (Hurxthal 1979).

1.2 Mating

1.2.1 Male

In *S. c. massaicus* both the territorial males and major females have a high parental investment (effort). The territorial male investment starts with nest building. He makes several scrapes on the ground one of which is selected as a nest by the major female. Both the territorial male and the major female incubate the eggs (males 67% of the time (approximately 0900 to 1700 hrs) and females 33% (approximately 1700 to 0900 hrs) (Hurxthal 1979), brood and escort the young. Probably as a consequence of male parental investment (Trivers 1972), males also seem to exert some mate choice (Hurxthal 1979). Territorial males initiate courtship by displays but occasionally refuse to mate with some hens visiting their territories (Hurxthal 1979, Bertram 1980). Females respond with a solicitation display characterised by approaching the male with lowered and quivering wings (Hurxthal 1979, Bertram 1979, personal observations). Male refusal is characterised by the following behaviour: courtship display (formal approach, song, kantle (Chapter 3) but on closer examination of the hen the male turns aggressive and chases her off. The male may also loose interest without aggression (Hurxthal 1979). Factors that may affect male mate choice in species where the male offers resources include favouring the most fecund

female (Parker 1970, Trivers 1972, Forsberg 1987) and discrimination of individuals based on recognition and events (Hurxthal, 1979). Territorial male ostriches copulate frequently with females on their territory and also preferentially with any new female visiting their territory (Hurxthal 1979). Hurxthal's data however, did not clarify which female (i.e. the major or the minor female) the male was repeatedly copulating with. Out of 16 courtship displays observed by Hurxthal, 13 were directed towards females the male was not accompanying. Female ostriches were observed to mate at least twice a day with different males (Hurxthal 1979).

The operational sex ratio may also contribute to mating choice. Territorial males are scarce (1:3 territorial males to breeding females (Table 3.2, Hurxthal 1979). This gives the male a choice of many hens). It is not clear why males preferred to establish territories in particular locations. Disfavoured areas at Nairobi National Park did not seem to have any obvious habitat inferiority (Chapter 3, Hurxthal 1979).

1.2.2 Female

Females only mate with territorial males within the males' territories. They do not mate with non-territorial males or territorial

males outside their territories (Hurxthal 1979). Acceptance of a soliciting male is signaled by squatting and allowing copulation to proceed. Refusal is by walking or running away (Hurxthal 1979, personal observations). Female preference for males with good territories increases male-male competition even in males exercising mate choice (Trivers 1972). Since nesting occurs within territories, the most obvious form of selection for good parental care is the inability of a non-territorial male to attract a female.

Females' (including major and minor females) home ranges overlapped 4-7 males' territories (Hurxthal, 1979). Since the males solicited copulations from the females as they came into the males, territories, the females would have these males to choose from. In birds multiple female matings have been explained in terms of benefits from material contributions of several males (Davies *et al.* 1996), genetic benefits for offspring (Kempenaers 1992), for assessment of future mates (Ens 1993) and being a response to possible mate's multiple mating; the female may attempt to monopolise matings as an attempt either to deplete the males sperm or decrease his chances of fertilising other females' eggs and therefore possibly decreasing resource competition for own offsprings (Birkhead & Moller 1992).

1.3 Communal nesting

Ostriches have a unique communal nesting system comprising several females laying in a single nest. The major female makes no attempt to stop the other females from laying in her nest (Bertram 1992). Only the major female and the territorial male incubate the clutch and escort the brood (Hurxthal 1979, Bertram 1979, Sauer & Sauer 1966, Jarvis & Jarvis 1985). In *S. c. massaicus* 4-17 minor females have been recorded laying in a nest (Hurxthal 1979) while the South African subspecies *S. c. australis* has commonly 2 minor females (Sauer & Sauer 1966). *S. c. massaicus* have clutches ranging from 29-67 while *S. c. australis* have smaller clutches ranging 16-23 eggs. The major hen contributes an average of 9 and 8 eggs in the *S. c. massaicus* (Hurxthal 1979, Bertram 1982) and *S. c. australis* (Sauer & Sauer 1966) respectively. The major female lays, on average, twice as many eggs in the nest as any other female (Hurxthal 1979) and is therefore expected to have greater investment in the nest than any other female. The clutch is partitioned into a central clutch of 20-21 eggs (Hurxthal 1979, Bertram 1982) and a peripheral one comprising the excess eggs. Only the central clutch is incubated. Surplus eggs are pushed away, by the major female, onto the periphery and do not develop (Bertram 1982). The communal laying

of a clutch too large to be incubated may not decrease her reproductive success provided the major female can ensure that her eggs are incubated. On the basis of colour, shape and pore pattern, Bertram (1982) concluded that the major female's eggs were more likely to be found in the central clutch than were other females' eggs. He identified one egg in each nest laid by the major female, then categorised the remainder in the nest as hers or others' on the basis of his matching the eggs. Of the putative major female eggs identified in five nests studied, only one was discarded onto the peripheral clutch. He proposed that the major female was able to recognise her eggs possibly using the same visual information. Here, I have set out to confirm this by parentage analysis of the central incubated eggs using molecular markers.

1.4 Pair Bond

A pair bond is established between a territorial male and the major female. The pair copulate, guard, incubate and escort the young with some repeating the relationship in subsequent years (Hurxthal 1979). Such behaviour can be described as a pair bond similar to that found in majority of monogamous birds species (Lack 1968). Males whose nests have been destroyed sometimes begin nesting again later, usually with the same major female (Bertram,

1982). Farm data also indicate that such pairs are by far the most successful in incubating eggs and hatching young (Bertram 1982).

1.5 Incubation

The territorial male and major female form a pair bond. They incubate the central eggs in turns for 42 days after which hatching occurs. The male incubates incubate between 1700hrs and 0900hrs and the female the rest of the time. This is probably an anti-predatory behaviour as the male is more conspicuous during the day than the female. There is an obvious increase in predatory risk to the male especially at dawn and dusk. The cost to the female may arise mainly as a result of less foraging time in the daylight hours since ostriches are diurnal feeders. However, there is a paucity of data on predation or foraging during the various life cycles of the ostrich.

1.6 Termination of breeding season

The chicks and adults leave the nest in 3-5 days after hatching the brood (Hurxthal 1979) by which time the yolk reserves of the chicks are exhausted (Smit 1963). The precocial chicks are escorted away by both parents and do not return to their nest after leaving.

1.7 Sex ratio

The ostrich adult sex ratio has been recorded as being slightly biased in favour of females, 1:1.11 at Nairobi National Park (Hurxthal) and 1:1.4 at Tsavo National Park (Bertram 1982). The operational sex ratio of territorial males to breeding females is 1:3 (Hurxthal 1979). This may explain why some females, unable to pair with territorial males, end up as minor females.

The ostrich adult sex ratio may be secondarily adjusted by factors such as adult differential mortality due to sexual dimorphism as a consequence of sexual selection or due to sexual bimaturation with females maturing at 3 and males at 4 yrs of age (Douglass, 1881). However, no data are available to support any hypothesis for secondary sex ratio adjustment. Alternatively, the sex ratio skew may be present at oviposition. Adaptive sex allocation (primary sex ratio bias at oviposition) has been demonstrated in birds (Lessells *et al* 1996, Appleby *et al* 1997, Komdeur *et al.* 1997). This has become possible due to the development of techniques that can sex offsprings even before hatching (Lessells *et al* 1996). Here I seek to estimate the primary sex ratio of the Nairobi National Park population as a probable indicator of adaptive primary sex allocation. I examined this by characterising foetal sex using an ostrich molecular sex probe.

1.8 Discussion

How does the ostrich resolve inter-sexual conflict brought about by a mating system that is mainly polygynous coupled with communal egg laying on the one hand and a monogamous parental care system on the other? Using molecular genetic markers, I examined the fertility consequences of the communal nesting system with reference to the territorial male and major female. Given the potentially long-term pair bond, does the major female mate monogamously with the resident territorial male who provides paternal care or does she seek extra-pair matings? Does the major female lose fitness by incubating eggs of other females at the expense of her own? The communal egg laying of a clutch too large to be incubated may not decrease the major female's reproductive success provided she can ensure that her eggs are selected for the central incubated clutch. The territorial male copulates repeatedly with females within his territory and preferentially with any new female entering his territory. Does he fertilise all the major female and any of the minor females' eggs? Minor females, make no parental investments after egg laying. Does the major female give access to minor females that are her close relatives hence accruing inclusive fitness benefits or are the minor females simply dumping their apparently inexpensive eggs to take advantage of the spare space

available in the nest? I investigated this by parentage analysis using molecular microsatellite genetic markers.

Chapter 2

THE SPECIES

2.1 Introduction

Modern living birds consist of 2 superorders: palaeognathae, comprising the tinamous and ratites, and neognathae which includes all other modern birds. The complete classification of modern living birds is a hierarchical arrangement of 29 orders and 187 families which include 2029 genera and approximately 9,600 species (Gill 1994).

Living ratite birds are the ostrich (*Struthio camelus*) of Africa, the greater rhea (*Rhea americana*) and lesser rhea (*Pterocnemia pennata*) of South America, the emu (*Dromaius novahollandiae*) of Australia, 3 species of cassowary (*Casuarius*) of Australia, and 3 species of kiwi (*Apteryx*) of New Zealand. Ratites and tinamous, on the basis of sharing a unique paleognathous (dromaeognathous) palate, have been grouped together into a single superorder paleognathae. Ratites are flightless with reduced wings and lack a sternal keel (Merrem 1813). There is fusion of the coracoid and sternum and the clavicles are absent or reduced. They possess a

grooved rhamphothecal structure, a penis and have loose plumage with poorly developed aftershafts except in emus and cassowary. They are also nidifugous.

2.2 Monophyletic or Polyphyletic ?

There has been controversy over placing the ratites and tinamous in the same superorder but separate from other birds. Furbringer (1888, 1902) viewed the palatal bones as a convergence. This was supported by McDowell (1948) who pointed out that the palaeognathous palate, though similar, actually consisted of four morphologically different forms and indicated convergent evolution. Storer (1971) asserted that grouping large flightless birds such as ostriches, rheas, emus, moas and elephant birds into one superorder was a holdover from pre-Darwinian classifications and not based on available evidence.

However, many workers support the opposite view. Evidence that ratites and tinamous should be placed in the same order, by virtue of being monophyletic, is supported by the lack of or reduction of the keel on the sternum (Merrem 1813), the presence of a paleognathous (dromaeognathous) palate (Huxley 1867), the structure of the rhamphotheca (Bock 1963, Parkes & Clark 1966), similarity of the axial skeleton (Mivart 1877), behaviour (Meise

1963) and mating systems (Handford & Mares 1985). Osteological (both cranial and post cranial) cladistic analysis has supported the monophyly of paleognaths. Molecular evidence either using DNA-DNA hybridisation (Sibley & Ahlquist 1990) or mitochondrial DNA (Cooper 1992, Lee *et al.* 1997) also supports the monophyly of ratites and tinamous. Paleogeographic evidence point to isolation and differentiation of ratites as a result of continental drift during the late Jurassic to early Tertiary periods, that broke up Godwanaland, eventually separating Africa, South Africa, Australia and New Zealand (Cracraft 1974). Certainly most authors now support the monophyly of palaognaths (Ho *et al* 1976, Prager *et al.* 1976, Rich 1979, deBoer 1980, Sibley & Ahlquist, 1990, Stapel *et al.* 1984, Cracraft 1981 1986 1988, Bledsoe 1988, Cracraft & Mindell 1989).

The question has now moved on to the phylogenetic relationship among the ratites.

2.3 Phylogenetic relationships

There are disparities in the phylogenetic relationships of palaeognaths. Cracraft's (1974) osteological examination placed the tinamous basal to the ratites, rheas and ostriches as terminal sister taxa as were cassowaries and emus. Bledsoe's morphological analysis was similar but placed kiwis as a sister group to the emus

and cassowaries. DNA-DNA hybridisation yielded an identical topology to that of Bledsoe's (1988) or depending on the clustering algorithms assumptions, placed the ostrich basal to the other ratites (Sibley & Ahlquist 1990). Sequences from a small fragment of the mitochondrial DNA 12S rRNA placed the rhea at the basal position. Questions then arise: Are rheas and ostriches allied more closely within the ratites? Is the kiwi a sister group to the emu and cassowaries or basal to the other ratites? If the former is true, how is the reversal to primitive morphological characteristics explained? Lee *et al.* (1997) set out to investigate this by combining both a large molecular data set of 5444 base pair mitochondrial sequences and 58 osteological characters covering both cranial and post-cranial elements. Complete nucleotide sequences of the Cytochrome B genes, 16S rRNA, $tRNA^{lys}$ and large portions of 12S rRNA, Cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes, and a 12S rRNA gene fragment (Cooper 1992) were used. Using tinamous, galliforms and anseriforms as outgroups (since galliforms and anseriforms are basal to their taxa in neognaths; Cracraft 1988, Sibley & Ahlquist 1990), derived character states were used in phylogenetic analysis. The most parsimonius tree, combining both morphological and molecular data (Figure 2.1) agreed with Cracraft's (1974) and, with the exception of the kiwi as sister group

to the emu and cassowary, is in agreement with Bledsoe's (1988) morphological analysis and Sibley & Ahlquist's (1990) DNA-DNA hybridisation data. These place the kiwi basal to other ratites and are consistent with the primitive morphological character states of the kiwi. The ostrich and rheas are closely allied as are the emu and cassowaries.

2.4 Ratites: primitive or derived within birds?

Irrespective of monophyletic or polyphyletic origins, earlier workers considered the Paleognaths to be primitive within living birds (Fulbringer 1888, Pycraft 1900, Lowe 1928) even though, conversely, they accepted that ratites descended from primitive flying ancestors. Ratites have anatomical features that can only be explained as adaptations for flight (Pycraft 1900, DeBeer 1956): fusion of wing elements (carpometacarpus), presence of a pygostyle, cerebellar structure and the presence of an alula on the wing. The paleognathous skull appears advanced whereas the neognathous condition is primitive (Cracraft 1974). Many feathers of the pelvis and the leg skeleton can be derived from galliforms and other non-pessarine families (Cracraft 1974). Evidence seems to point to ratites being relatively advanced both morphologically and probably phyletically. Mitochondrial DNA analyses have nested the ratites within the

neognathae an indication that the ratites are very recently derived from neognathae, possibly by neoteny (Harlid & Arnason 1999). However this was a limited study that only examined rheas and ostrich; therefore further studies need to be done to gain better resolution.

Since the consensus is that the ratites and tinamous appear to be derived within birds and are monophyletic, classification into a separate taxa such as the order Paleognathiformes (Figure 2.2, Cracraft, 1981) or infraclass Eoaves, seems most logical (Figure 2.1, Sibley & Ahlquist). However, the term paleognathae resulting from early assumptions of primitiveness seems inappropriate and should be applied with caution. Indeed, current generally accepted classifications avoid the superorder paleognathae (Figure 2.3,Welty 1983, Storer 1971) but by placing the ratites and tinamous in the superoder neognathae, the authors imply polyphyletic origins. With the current morphological and molecular evidence, this classification should be amended.

2.5 Tinamous and ratite breeding system

The tinamous and ratites show an unusual parental care system that is prominently or exclusively paternal. This is associated with an array of mating systems ranging from monogamy to

polygyny/polyandry (Handford & Mares 1985). The ostrich has a polygynandrous mating system coupled with biparental care (Hurxthal 1979, Bertram 1980), a unique and unknown system among higher vertebrates outside the paleognaths. However, it is similar to that of the rhea (Bruning 1974) with the modification of possessing biparental care. To appreciate the adaptive nature of the ostrich breeding system, it is imperative that the correlation between paleognath mating system and phylogenetic relationship be fully understood.

2.6 Classification

Figure 2.1: Classification of ratites and tinamous into a separate subclass based on DNA-DNA hybridisation studies by Sibley &Ahlquist, 1990.

Subclass Neorthines

Infraclass Eoaves

Parvclass Ratitae

Order Struthioniformes: Ratites

Suborder Struthioni

Infraorder Struthionides

Family Struthionidae: Ostrich

Infraorder Rheides

Family Rheidae: Rheas

Suborder Casuarii

Family Casuariidae

Tribe Casuariini: Cassowaries

Tribe Dromaiini: Emus

Family Apterygidae: Kiwis

Order Tinamiformes

Family Tinamidae: Tinamous

Figure 2.2: Morphological classification of ratites and tinamous in a separate order paleognathiformes as postulated by Cracraft, 1981.

Order Palaeognathiformes

Suborder Ratiti

Infraorder Struthiones

Superfamily Struthionoidae

Family Struthionidae: Ostriches

Family Rheidae: Rheas

Superfamily Casuarioidae

Family Casuariidae: Cassowaries

Family Dromiceidae: Emus

Infraorder Apteryges

Family Dinornithidae: Moas

Family Apterygidae: Kiwis

Suborder Tinami

Family Tinamidae: Tinamous

Figure 2.3 Classification of ratites and tinamous into a separate subclass Neonithes based on morphological, molecular and behavioural evidence (Storer 1971, Welty 1982)

Subclass Archaeonithes (extinct)

Subclass Neornithes

Superoder odontognathae (extinct)

Superoder neognathae

Order Struthioniformes

Family Struthionidae: Ostrich

Order Rheiformes

Family Rheidae: Rheas

Order Casuariiformes

Family Dromiceidae: Emus

Family Casuariidae: Cassowaries

Order Tinamiformes

Family Tinamidae: Tinamous

Order Dinornithiformes

Suborder Apteryges

Family Apterygidae: Kiwis

Suborder Dinornothes: Moas (extinct)

2.7 The species

The Order Struthioniformes possess two distinct toes, the third and fourth digit. The third digit is strongest, supporting greater part of the weight. The body feathers are single with no aftershaft and the wing ("remiges") and tail ("retrices") are large but soft and plumose.

The ostrich, the largest living bird, is represented by the single genus *Struthio* with 4 extant and one recently extinct subspecies or races; *Struthio camelus camelus* and *S. c. syriacus* (extinct) of West and North Africa, *S. c. massaicus* of East Africa, *S. c. molybdophanes* of Somali land and Central Africa and *S. c. australis* of South Africa.

The males are larger than the females, standing about 2.1 meters to the tip of the head and 1.5 metres to the back. The exposed neck and hind limbs in *S.c. camelus* and *S.c. massaicus* have a pale fleshy colour turning bright pink in the breeding season. This may, as observed in other birds, be due to increased blood flow resulting in transient colour changes (Hurxthal 1979). Males acquire a black and white adult plumage at the age of 1 year, about a year before demonstrating the pink flush and breeding behaviour (Hurxthal 1979). In *S.c. molybdophanes* and *S.c. australis,* the male skin coloration is grey-blue and they in addition possess red tarsal scales

S.c camelus and *molybdophanes* have a horny shield on the crown lacking in *S.c. massaicus* and *S.c. australis*. In all the subspecies, female and juvenile male plumage is grey.

The subspecies, found exclusively in Africa, are geographically isolated with the exception of *molybdophanes* and *massaicus* of East Africa which overlap in Laikipia and Tsavo west (Bertram 1992). A belt of brachystegia woodland effectively divides the ostrich into northern and southern populations (Hamilton 1982) with the former incorporating the *camelus*, *molybdaphanes* and *massaicus* while *australis* is confined to South Africa (Brown *et al* 1982). The Ethiopian system of the Rift Valley separates *camelus* from *molybdophanes* and *massaicus*. Farm data however, show that the subspecies can interbreed under artificial circumstances producing viable offspring (Hurxthal 1979). Using mitochondrial DNA restriction fragment length polymorphisms (RFLP's), Freitag & Robinson (1993) showed restricted gene flow between *camelus* and the East African subspecies. This parallels the geographical isolation of these 3 subspecies by the great Ethiopian Rift Valley (Freitag & Robinson 1993). Interestingly, decreased gene flow was also evident between *molybdophanes* and *massaicus*. This is probably as a result of different ecological and behavioural/reproductive cues. *Molybdophanes* readily enters bushed regions and is a browser while

massaicus is restricted to open savanna and is a grazer (Jackson 1938, Lewis & Pomeroy 1989) and interbreeding barrier such as differences in courtship displays have been reported between them (Jackson 1938, Lewis & Pomeroy 1989). Indeed some workers argue that given the phenotypic, behavioural, ecological and mitochondrial DNA divergence, a species status for *molybdophanes* may be warranted (Jackson 1938, Lewis & Pomeroy 1989, Freitag & Robinson 1993). Indeed *molybdophanes* has been described as a separate species (Ogilvie 1905). RFLP analysis has revealed evidence of gene flow between *australis* and the East African subspecies. This is probably due to recession of the brachystegia woodland and connection via an arid corridor (Moreau 1966, Verdcourt 1969, Hamilton 1982 and Kingdon 1990) the most recent probably occurring approximately 20,000 to 12,000 y.b.p (Hamilton 1982).

Chapter 3

FIELD STUDY AND SAMPLING AT NAIROBI NATIONAL PARK.

3.1 Introduction

The study area was Nairobi National Park. The park is less than 10 kilometers from the city center and covers an area of 117 km^2 (Figure 3.1) and lies at 1,800 m above sea level. The park's richness in fauna and flora is exhibited by over 100 mammal 400 bird species. This species richness results from habitat heterogeneity (Stanley Price 1974) within the park and the fact that the park is a dry season concentration area, with better quality forage and water resources during the dry season. As much of East Africa has variable rainfall with wet and dry seasons (Griffiths 1958), dry season concentration areas such as Nairobi National Park have abundant migratory and endemic species.

Habitats in Nairobi Park range from forest to savanna grassland: from rocky gorges and escarpments to rolling plains. Three factors contribute to this diversity – change in elevation, rainfall gradient and underlying geology. The elevation ranges from 1,800m above sea level at the western end of the park to 1,500m at the

eastern end. Related to the topographic gradient is rainfall with an annual mean of 900mm at the west end and 600mm at the eastern end (Hurxthal, 1979).

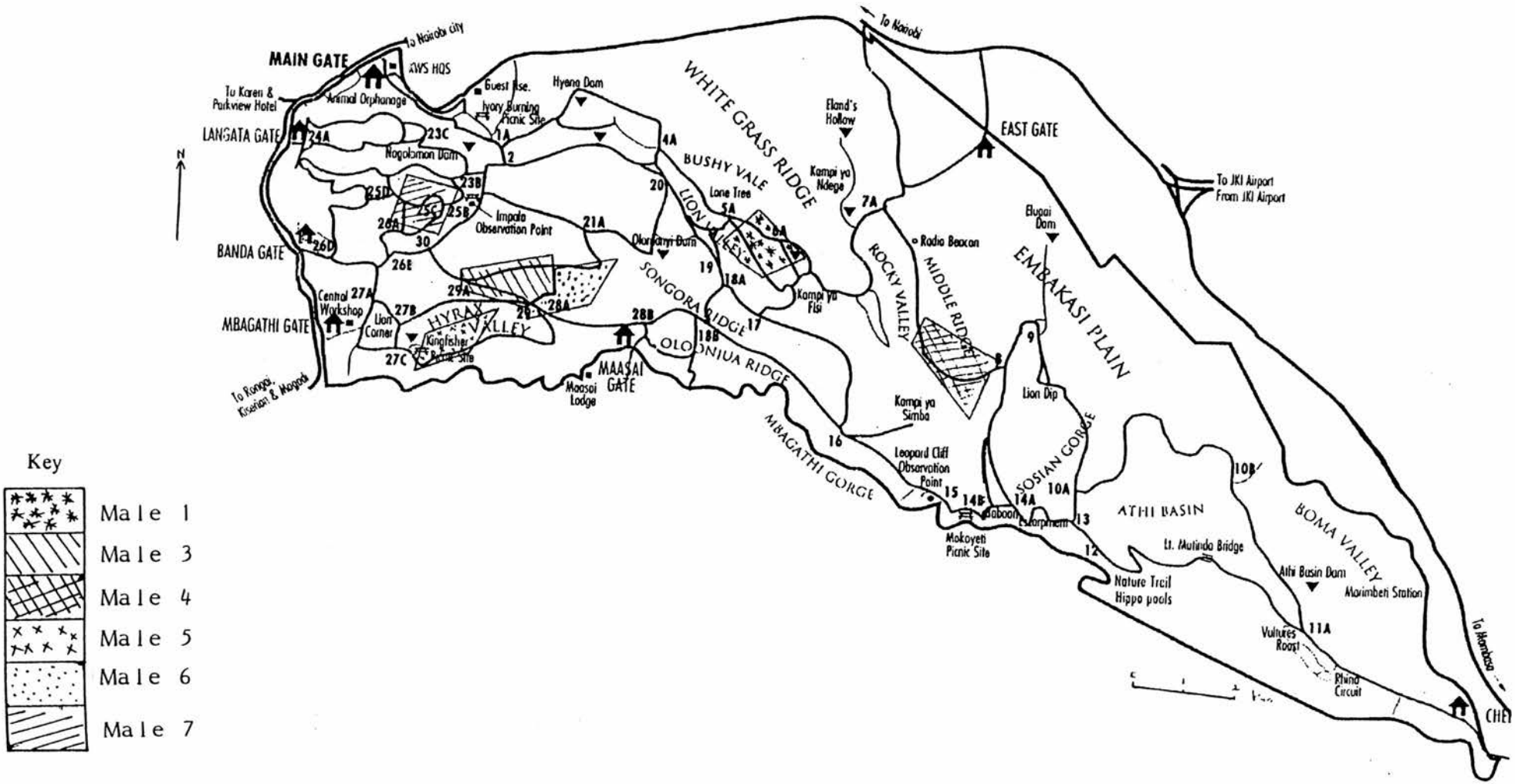
The species richness of this sanctuary led to lobbying that resulted in gazetting of Nairobi National Park as the first national park in East Africa on December 16, 1946.

Increased human activity and growth of Nairobi has led to fencing off the north, east and west by a chain link and an electric fence to prevent animals straying into the city. However, the southern end is left open to allow migration of animals.

We chose Nairobi National Park as the study area because of ease of communication both within and without the park, abundance of our study species (a total population of 188 ostriches, Kenya Wildlife Services census, 1997) and the fact that Hurxthal (1979) used the same area to study behavioural aspects of the *massaicus* ostrich breeding system, a study that motivated us to follow up using newly developed molecular markers to examine the parentage in this unique breeding system.

Hurxthal's (1979) field study was between 1971 and 1973. Of the 1978 counts in a 10 - month period, 15 breeding pairs hatched and led 152 chicks from their nests in 1971. He catalogued background information on social communications in which 18 major displays

Figure 3.1 Territories of six territorial males at Nairobi National Park



and 25 non-ritualised social signals were used. Part of these, described in section 3.2.2.2 below, formed the main breeding behaviours encountered in my field study.

Five aspects of Hurxthal's (1979) study provided information on the dynamics of breeding at the individual and population level: 1) Group size – five to nine adults of both sexes were typical outside the breeding period reducing to three to four individual with many solitary individuals during the breeding season. Cocks became territorial and females moved among territories alone or in groups. 2) Social spacing – cocks were territorial, holding territories of 2 ± 0.9 (SD) km^2 while females had home ranges three times that size. 3). Mating system – Though both sexes typically mated with more than one of the opposite sex, mate choice was commonly observed. However, each territorial cock had a pair bond with a single major hen lasting for one or more seasons. Only the pair provided parental care comprising egg guarding, incubation, brooding and escorting the chicks. 4) Communal laying – An mean of 7.4 ± 4.1 (SD) females, comprising one major female and minor females laid a mean of 45.1 $\pm$ 17.2 eggs per nest. Only the major hen and the territorial cock incubated the eggs, with the major hen pushing out eggs in excess of

21. 5) Creching – Synchronous breeding and progressive brood merging resulted in a large single creche comprising all or most of the brood in the population and escorted by just a few of the original parents.

Objectives

The fieldwork objectives were:

1. To monitor the breeding ostriches in the 1997/98 season.
2. To collect tissue samples from the breeding pairs and their offspring.

3.2 Methods

3.2.1 Field observations.

Ostrich breeding behaviours have been described in detail by Hurxthal (1979) and Bertram (1992). My observations restricted to the nesting individuals i.e territorial male, major and minor females. The emphasis in my field study, which was restricted the 1997/98 season, was collection of tissue samples for molecular analysis.

3.2.2 Major ostrich behaviour during breeding

We observed the following behaviours displayed by the ostriches during breeding as described by Hurxthal (1979).

1. Skin flush.

   The normally flesh coloured skin of the legs and neck turn pink, probably due to a mechanism of epidermal blood vessels. Usually evident in May through to October or November.

   It is a common and visually channeled display, possibly directed at both sexes. Displayed at the beginning of the breeding season though the specific environmental stimulus is unknown. This display probably initiates female breeding behaviours.

2. Hiss or open beak threat.

   Often performed when individual are threatened either due to invasion of individual distance by other ostriches or predators. It is a common acoustic and visual display. It is a hoarse note either uttered with beak open once or continuously during aggressive encounters (e.g. distraction display when escorting chicks or in fights)

3. Head low

   Is a visual and common display. Cocks direct it to cocks and females to either sex. Is a submissive posture when a hen approaches a cock, or preparing to lay in a nest with another female already present or when an individual is approaching a group of adult ostriches.

4. Wing flapping

Is a visual and common display performed by cocks but directed at both sexes. It is an aggressive display commonly seen in male-male interactions.

5. Formal approach

Is occasionally performed by cocks and directed to both sexes. Is a visual display starting off a series of courtship display if directed to a female but is also used to approach a male intruder aggressively. The tail is held back or slightly raised, neck kept straight with head moving backwards and forwards. The black neck feathers become erected as may the body and tail feathers.

6. Kantle

Is a visual and acoustic display occasionally displayed by cocks and directed to males in male-male aggression or females in courtship sequence indicating copulatory interest. With the male lying down or raised at the hocks, the wings are spread out and alternately raised and lowered with the neck and head inclined posteriorly and sweeping the back, in opposing direction to the wings, with a soft rhythmic thump.

7. Full Threat

Is an occasional visual display performed by cocks to females the last in a series of courtship displays preceeding copulation and

to males during aggressive male-male encounters. It consists of a formal upright advance with wings raised and primaries fully spread. Lasts about 8 seconds and is usually performed at a walk but occasionally at a slow run. Probably serves as a signal proposing the female to lie down in preparation for mounting. Directed towards males this signaled an escalation from threat to fighting.

8. Soliciting

   A visual display that was common in females but rare in males that is stimulated by the presence of an individual in breeding condition. With the bird standing, the wings and primaries are spread letting the wing fall in a gentle curve outward and downwards. They vibrate repeatedly and the head is also lowered and raised repeatedly. The response to this behaviour may be aggression with rejection or courtship and copulation.

9. Copulation

   The male approached the female in quick small steps. The male would then place his right foot on the female's back and the left on the ground before dropping gently onto her back. Penile penetration then follows and the male then begins a smooth swing of his neck and wings identical to the kantle. The swings slow down, the wings and body vibrate and a deep rhythmic grunting

vocalisation is emitted with bill snapping and feather erection. The behaviour suddenly stops after about 1 minute.

10. Nest site scraping.

Was a visual and rare display by the males directed towards females. The male lay down and scraped alternately with his legs with or without accompanying females. Some of these scrapes were selected as nests.

11. Song.

Is an occasional acoustic display by males dirrected at both sexes. Used when male first sighted a female as one of the first displays in courtship display or in the absence of hens probably to advertise breeding condition, specific location and territory. Was also used in male-male aggression. It consisted of three woo woo woooo notes lasting three to four seconds (repeated 1 to 6 times) and could be heard up to a kilometre away. The notes were of the same pitch and loudness with the last longer than the previous two

3.2.3 Field Methods

Individual identification was difficult as the birds' morphological differences were not easily discerned. The females

were especially difficult as they were a cryptic grey brown colour with no distinctive features. In the end, neither males nor females were individually identifiable. Therefore we coupled individuals to a particular nest. We surveyed as large an area as possible daily using the *ad libitum* method (Altmann 1974) to cover a broad spectrum of observations. Focal animal sampling was used after identification of nesting individuals. The individuals were then tracked for as long as necessary enabling focal animal observations, territory estimation and subsequent biopsy sampling. Given the savanna plains, the fact that the ostrich is a large cursorial grazer and the well-distributed park roads, the focal method was ideal for coupling individuals to nests and recording their behaviour. We used a 20-70X telescope (Kowa®) and 10X binoculars (Zeiss®) to record the observations from within or atop a clearly marked pick-up truck. This was a requirement by the KWS intended as a signal to interested parties that we were venturing off road for research purposes. Movement off-road is strictly forbidden to tourists. Even so, we had to be very discrete when venturing off-road since tourists would be attracted to the nest and so expose it to an increased risk of predation/desertion.

3.2.4 Breeding interactions and territory estimation

Park regulations forbade us from leaving the confines of our pick-up except in the presence of KWS personnel. Breeding observations were therefore made from the pick-up with the telescope mounted on the window. We could scan up to 2 km with good resolution. The male territories were mapped by observing a male from the time of change-over of incubation with his female at approximately 0900h, to the time it took over again at approximately 1700h. The mapping was carried out after incubation started. Due to time constraints, we were unable to observe any given male for continuous stretches longer than one full day. The information gathered, along with other shorter observations was used to estimate the territory size from the furthest points a male was observed foraging from his nest from the time he left the nest to the time of his return.

3.2.5 Nests

Nest discovery was a very arduous task as females are cryptic against the grassland habitat background and easily concealed themselves by lowering their necks. Thus six of the eight nests were discovered during incubation (with the exception of nests 3 and 8)

which were chanced upon as a female was laying in them). At this time, either the major hen or the territorial male was continuously on the eggs. A female completes the laying of an egg in 1-2 (Hurxthal 1979, personal observation) minutes. This makes it virtually impossible to find the nest before incubation. An early nest could be recognised by the presence of few eggs and initial slow growth due to continued laying, by the major female, of one egg every two days. A rapid clutch growth phase of over 2 eggs per day then followed presumably due to discovery of the nest by other females (Hurxthal 1979) Once the eggs were partitioned into central and peripheral clutches, the nest was at an incubatory stage.

3.2.6 Field Period

June – August

Once-weekly visits to the park were carried out to identify initiation of the breeding season. Large social groups of 7-10 individuals of mixed sexes diminished to small groups comprising mainly of a few or single females in the company of one male in breeding condition. When a male was ready to establish a territory and breed he became increasingly aggressive and could be identified by a pink flush on his bare neck and legs.

Late August – mid-September

During this period extensive *ad libitum* field observations were carried out. Daily coverage of as much of the Park as possible was carried out from between 0800 to 1800h and nest locations occurred. By deduction from the earliest nest hatched (which was not among those monitored), laying and incubation started approximately between early to mid-August and early to mid September respectively. Six nests were discovered on mid- to late-September. All the nests were discovered as a result of locating a sitting female. Nest 1 was exceptionally discovered due to 5 females exhibiting hiss displays on the nest, making them highly conspicuous from a distance of at least 2 km away.

Mid-to end-September

7 of the 8 nests had been discovered by this time. An initial count of eggs was done as soon as a nest was located, taking note of any partitioning into a central and peripheral clutch. Thereafter, each nest was observed at least twice a week from the nearest dirt road/track (20-200m) and visited weekly. Nest visits entailed driving off-road to the nesting site. The incubating birds normally got up and retreated to a distance of about 10-20 metres. We then counted the nests and noted the number in the peripheral and central clutch before

leaving the nest. In every case, the nest was incubated again within 10 minutes of our leaving the site.

October – November

As incubation proceeded, daily observations were carried out. We intended to take samples of the chicks' tissue and this had to be done while the chicks were still on the nest. Since the chicks are escorted from the nest within 2-4 days of hatching, a visit to the nest every other day was necessary. On 19/10/97, unusually heavy rains began as a result of the *El Nino* effect. Females were thereafter observed to sit next to the nests presumably to allow drying off before continuing the incubation. This strategy seemed to work initially, as breaks in rainfall allowed drying out. However, the rains became increasingly heavier resulting in the first nest, nest 4, being abandoned on 22/10/97. To salvage the situation, we approached KWS who on examination of the nests gave us permission to collect eggs on 24/10/99. The other nests were also gradually abandoned and by 6/11/97, permission had been granted to collect eggs from 5 nests. The eggs collected were from nests 3, 4, 6, 7 and 8.

3.2.7 Collection of tissue samples from breeding pairs and offspring

The adult birds were positively identified by coupling them to a nest as earlier described. A small skin sample of approximately 0.1g was collected using a biopsy dart (Palmer®) from the territorial male and the major female. The darting, using a gun charge, was carried out by a KWS veterinarian in accordance with Park regulations. The dart dropped off after hitting the bird and a skin sample was left attached to a barbed central dental probe. The sampled bird would then be left undisturbed for the rest of the day. No desertion resulted as a direct consequence of biopsy collection. In fact the birds seemed to hardly notice and only stepped away.

We intended to sample chicks by plucking off a few feathers within 48 h of their hatching and while they were still in the nest. However, flooding and abandonment of the nests meant that we had to resort to collecting eggs within the central clutch, along with a few peripheral eggs. The eggs were transported to The Department of Animal Physiology, University of Nairobi where they were frozen until tissue harvesting. Chick tissues (mainly the egg membrane) were collected, frozen and transported to the University of St.

Andrews, Scotland, where DNA extraction and analysis was carried out.

3.3 Results

In the entire park, 23 potential breeding pairs were found. A territory was recorded if one male, in breeding condition, was in the company of one or several females. If two or more males were seen in the company of a female(s) and one exhibited aggressive behaviour such as threat approach, kantling and a chase, this was included as a possible breeding pair. Most of these pairs were found in the central areas of the park covering the Hyrax Valley, Songora Ridge, Lion Valley and Rocky Valley. Surprisingly few potential breeding pairs were found in the grassland ridges of White Grass Ridge and the Athi Plains despite their apparent suitability as potential breeding areas (Figure 3.1). Hurxthal (1979) also noted that certain areas were ignored as territories by males even though there was no obvious qualitative difference between them and the preferred sites. Groups of breeding individuals were logged in as different if they were sequentially observed on the same day or if they were observed on different days at least 2 km apart. 23 territorial males and 67 females ranging within their territories were identified, a 23:67 (1:2.8) territorial males:adult females ratio.

3.3.1 Nests and biopsy sampling

In total, 8 nests were located (Table 3.1). Of these, nest 5 was destroyed by predators 12 days after we had discovered it. Nests 1 and 2 were abandoned. No samples were collected because parental tissue samples were unavailable. Adult biopsy collection was not always successful. The birds in nest 1, after an earlier failed attempt, learned to avoid us and would immediately move out of range when we appeared on or about the nest. Nest 2 belonged to a female that was apparently attempting to incubate on her own. No male was ever observed on the nest and she was seen incubating unusually early at 0700hrs and unusually late in the evening at 1830h. She would occasionally leave the nest during the day and forage nearby before resuming incubation. She eventually abandoned the nest. Though tissue samples had been collected from her, we chose not to collect her eggs, given the constraint of collecting only 5 nests' eggs, as we lacked her partner's sample. Since the nests were all abandoned before we began collecting the eggs, some predation occurred across most nests. Nests 3, 4, 6 and 7 lost 6, 2, 4 and 14 eggs respectively (Table 3.1). Only nest 8 did not lose any eggs to predators. Unfortunately this was the youngest nest and incubation had not yet

started, so when the eggs were opened no embryonic tissue could be found in any of these eggs. The total number of eggs per nest ranged from 18 – 66, with the peripheral clutch ranging from 1 – 40 eggs. Table 3.1 gives a breakdown of the fate of nests that we observed during the 1997/98 breeding season. Some peripheral egg samples were also collected. These were expected to have close to zero development as they were not incubated and had been exposed to the elements for prolonged periods. We therefore collected only a few to confirm this. The results agreed with our assumptions as none of the peripheral eggs had undergone any development and there was no embryonic tissue.

3.3.2 Territorial males

Six territorial males were extensively studied and their territories estimated. These were males from nests 1, 3, 4, 5, 6 and 7 (Figure 3.1). A territorial male, within his territory, was very aggressive towards other males but not females. A typical encounter as recorded for male 3 on 6/10/97 is cited :

Table 3.1: Fate of eggs, by the end of the study period , within nests monitored in the 1997/98 breeding season at the Nairobi National Park.

Nest	Central clutch					Peripheral clutch		
	Total	Predated	collected	Fertile	Infertile	Total	collected	Infertile
1	26	0	0	n/a	n/a	40	0	n/a
2	20	0	0	n/a	n/a	28	0	n/a
3	26	0	26	18	8	12	8	8
4	19	4	15	13	2	18	5	5
5	24	24	0	n/a	n/a	15	0	n/a
6	27	3	24	18	6	14	5	1
7	24	12	12	12	0	1	1	1
8	17	0	17	-	17	1	1	1

"After being relieved by the female at 0830h, the male foraged along the eastern end of his territory. He encountered a male who displayed aggressively towards him by kantling (Hurxthal, 1979) (squatting and rowing wings from side to side with the neck flexed backwards). This male then got put up, and following a full threat approach escalated the conflict into a chase. The chase proceeded for about 100 metres along the territory boundary. The territorial male then turned and reciprocated the challenge with a full threat approach. The intruder this time ended his aggressive display, and seemed to concede by turning away and retreating. The territorial male later encountered a second intruder who turned away without any threat behaviour being exhibited. A third male intruder was aggressively approached with a kantle display and a chase lasting for approximately 3 - 4 kilometres before the male turned back and headed for his territory. A female foraging within his territory was not given any attention, neither did the male attempt to mate with her." Males therefore aggressively defend their territory against other male intruders.

The minimum male territory sizes were estimated to be 1.0, 1.1, 1.5, 1.4, 0.8 and 1.0 km^2 for nests 1, 3, 4, 5, 6, and 7 respectively (Figure 3.1), a mean of 1.13 ± 0.26 km^2.

3.3.3Females

The female study was limited to observations of those that were laying in territorial males' nests. Extensive observations of the major females were not feasible as they nested during the daylight hours. Females were difficult to identify individually due to their cryptic appearance; this made them difficult to spot and track. Several females were observed to lay at any given nest. The highest number of females seen visiting a nest was 6 within $1^1/_2$ hours, with two of these females laying on the nest during this observation period. A female about to lay would exhibit a swollen and reddened cloaca. If other females were present, laying would be preceeded by jostling for nest position with hissing and elevated wing displays. The female would then sit on the nest and proceed to deposit an egg in 1-2 minutes. A mucoid cloacal discharge would be evident once the female got up. The female may then walk away from the nest and another female, presumably the major female, would proceed to manipulate the eggs for up to 10 minutes at a time. During incubation, and the time of take-over from the male, the female would always spend time manipulating the eggs before sitting on them. We did not witness a male manipulating eggs on any nest where he took over. It may be that they did not do so or they that they did this under cover of darkness when we were unable to observe it.

Chapter 4

DEVELOPMENT OF MICROSATELLITE MARKERS IN THE OSTRICH *Struthio camelus massaicus* (Kimwele *et al* 1998)

4.1. Introduction

Molecular techniques have opened up new perspectives in biological research in the last 20 years. DNA-DNA hybridisation has helped reconcile avian systematics (Sibley & Ahlquist 1990), mitochondrial DNA patterns have led to better understanding of genetic differentiation among populations and species (Kessler & Avise 1985) and randomly amplifying DNA fragments have been used to resolve parentage (Quinn *et al.* 1987). However, it is the application of minisatellite sequences in DNA fingerprinting (Burke & Bruford 1987, Wetton *et al.* 1987) that proved most sensitive in determining genetic relationships between individuals (Burke 1989a 1989b). DNA fingerprinting however needs large amounts of good quality DNA, 5μg per single individual per lane and putative parents and their offspring must be run concurrently on the same gel. Large tissue samples require prompt and adequate storage often difficult in field conditions. In natural populations, invasive sampling is beset with ethical and safety risks. The isolation of locus-specific

microsatellites have simplified such studies by allowing the use of very small amounts of DNA from tissue samples such as hairs and feathers. Such samples are relatively easy to collect and store under field conditions. Microsatellites are also locus-specific and hence allow independent genotyping of individuals. It is therefore not surprising that since their early application in genome analyses (Litt & Luty 1989, Tautz 1989) microsatellites have been widely used as genetic markers.

Microsatellites are mono-, di-, tri- or tetranucleotide repeats that are highly abundant in the genome. $(TG)_n$ number in the order of 10^5 in mammals (Hamada *et al.* 1982) on average occurring every 30 kb in humans (Stallings *et al.* 1991). Considering every possible motif, there is probably 1 microsatellite for every 6 kb in man (Beckmann & Weber 1992). Although their evolutionary conservation suggests some functional or structural significance such as regions of recombination (Pardue *et al.* 1987), gene regulators (Hamada *et al.*1984), stimulating chromosomal packing and condensation (Stallings *et al.* 1991) or coding regions of genes (McCaffery *et al.* 1997), there is no conclusive evidence of any function.

The hypervariable nature of these markers makes them highly suitable for identity or parentage testing (Morin and Woodruff 1992,

Ellegren 1992). In birds, since first isolated in barn swallow and pied flycatcher (Ellegren 1992), microsatellites have been widely applied in avian research.

Microsatellites however do have some pitfalls that may limit their resolving power. One is the occurrence of non-amplifying or null alleles and the other is linkage between marker loci. Occurrence of null alleles may lead to mismatches in parentage inference due to incorrect genotype assignment from the gel genotype e.g. A- being interpreted as AA instead of AO. Null alleles can be detected by following the segregation of allelic variants in family pedigree and looking for cases of uniparental inheritance (Callen *et al.* 1993). A locus exhibiting a common null allele should be excluded from analyses. If pedigrees are unavailable, as is commonly the case in natural populations, the frequency of null alleles may be estimated from the observed genotypes (Summers & Amos 1997) or may be indicated by observed deviations from Hardy-Weinberg expectations in the form of heterozygote deficiency (Chakraborty *et al.* 1992). Linkage between loci i.e when the recombination distance between a marker is less than 50 cM, may lead to linkage disequilibrium and the resolving powers will be lower than the product of individual powers per locus (Primmer *et al.* 1995). It is therefore useful to test for linkage disequilibrium test on marker sets. Practically, however,

increasing the size of marker set limits this problem by increasing the sensitivity of the marker system (Primmer *et al.* 1995).

The ostrich has a unique breeding organisation based around a communal nesting system. To understand its development and maintenance, it is necessary to measure the interacting individuals' reproductive success. I set out to achieve this by estimating parentage of clutches of eggs using microsatellite genetic makers. Microsatellite repeat sequences are ideal for this since they are highly polymorphic and can be used to genotype individuals from very small amounts of DNA (Primmer *et al.* 1995). I isolated polymorphic microsatellite repeats in the ostrich and developed specific sets of primers from their conserved flanking regions. Using genomic DNA as template, specific microsatellite loci were amplified by these primers using polymerase chain reaction (PCR). These specific loci, used to genotype individuals assuming a Mendelian inheritance pattern, are very useful in determining relationships between individuals in a population. Since only small amounts of DNA are used such as would be found in feathers or the chorioallantoic membrane and blood left in hatched eggs, the invasiveness of sample collection is minimised.

4.2 Methodology

4.2.1 Sample collection for developing microsatellites

Farmed ostriches at Maasai ostrich farm and Kiserian Research Unit, Kenya, were physically restrained and 2-5 ml of whole blood taken from the brachial vein in the wing using a G18 needle. 0.5 ml of 0.5 M EDTA was used to prevent clotting and DNA degradation. The samples were then packed in ice and transported to storage at -20°C.

4.2.2 Extracting genomic DNA

DNA was extracted by chemical extraction using a 1% ionic SDS detergent lysis buffer (0.15 M NaCl, 50 mM Tris pH 8.0, 1 mM EDTA) to disrupt the cell membrane and proteinase K to digest cellular protein. This was followed by extraction using the phenol / chloroform method. A volume of phenol (pH 7.8-8.0 with 0.1 % hydroxyquinolone) equal to the sample was added. Mixing was then carried out on a rotator for 5 minutes followed by centrifugation for 5 minutes. The top clear aqueous phase was then decanted and transferred into a clean microguge tube containing a half sample volume of phenol and half sample volume of chloroform : isoamyl

alcohol (24:1 v/v) followed by mixing and centrifugation. The process was repeated with chloroform/isoamylalcohol. The DNA was then precipitated with 3 volumes absolute alcohol (ethanol) and 0.1 volume 3 M sodium acetate. The DNA pellet was washed with 4 volumes 70 % alcohol for salt removal. The DNA was then dissolved in sterile distilled H_2O stored at –20 °C until needed.

4.2.3 Construction of genomic DNA Library

4.2.3.1 DNA quantification

The quality and concentration of the DNA was tested by electrophoresis in a 1.5 % agarose minigel and comparing the DNA with known standards or by use of a DNA fluorometer (Hoeffer® TK DNA fluorometer).

4.2.3.2 Restriction Digestion

Using pooled genomic DNA from 5 males and 5 females, 60 µl (30ug) was digested with restriction enzymes *HaeIII*, *AluI* and *RsaI* (Gibco BRL®) in 10x buffer at 37°C overnight. The digestion generated fragments with blunt ended termini.

4.2.3.3 Extracting digested DNA and size selection

Digested DNA was extracted using the phenol/chloroform extraction described above. Testing the DNA for complete digestion was done in a 1.5 % agarose minigel. 0.8% low melting point agarose gel electrophoresis was carried out and a 123 bp DNA ladder (Promega®) was used for size selection. DNA in the range 250-800 bp was recovered by cutting of the gel, melting in TE (10 mM Tris pH 7.5, 1 mM EDTA) and extracting using phenol / chloroform.

4.2.3.4 Vector Cloning

The plasmid vector pBS KS^+ (Stratagene®) was used. The plasmid was digested with *Sma* I (Gibco BRL®) restriction enzyme which recognises the restriction site CCC/GGG.

In cloning, the major difficult is distinguishing between plasmids that contain inserted foreign DNA (recombinant DNA) and vector molecules that have recircularised without the insertion of foreign DNA. We limited recircularisation by adjusting the concentrations of vector:insert and thereby optimising the number of correct ligation products.

Since we used blunt ended foreign DNA termini, the efficiency of ligation was low due to high levels of background non-

recombinant clones. To increase efficiency, one would have to use high concentrations of DNA and ligase enzyme. Alternatively, condensing agents may be used. We used the condensing agent polyethylene glycol-8000 (a component of the 5x T4 ligase buffer GIBCO BRL®). Condensing agents increase macromolecular crowding and cause DNA molecules to condense in aggregates. These substances have the following effects on ligation:

1. Accelerate the rate of ligation of blunt-ended DNA by 1-3 orders of magnitude therefore reactions can occur at low DNA and ligase concentrations.

2. The distribution of ligation products is altered by suppressing intramolecular ligation. Ligation products are exclusively by intermolecular joining events. This then inhibits recircularisation.

Another disadvantage of using blunt ended termini is the possible elimination of restriction sites between the plasmid and foreign DNA.

4.2.3.4.1 Dephosphorylation

To inhibit self-ligation and recircularisation of the plasmid DNA, dephosphorylation was carried out to removed the 5'-phosphate group using CIP (calf intestinal alkaline

phosphatase)(Pharmacia®). The resultant 5' hydroxyl group was then incapable of forming a phosphodiester bond during *in vitro* ligation by bacteriophage T4 ligase (since this reaction requires a 5-phosphate group on one nucleotide and 3- hydroxyl group on the other). Ligation to foreign DNA (since this still possesses 5'-phosphate group) was possible albeit with single-stranded nicks on either side. These nicks were repaired on transformation into competent cells.

4.2.3.4.2 Ligation

Ligation of insert DNA into pBS was carried out using bacteriophage T4 ligase (Gibco BRL®) enzyme since it more efficiently joins blunt-ended DNA fragments under normal reaction conditions than *E.coli* ligase.

Different ratios of vector:insert (1:1, 1:2, 1:4 and 1:6) were used to optimise the number of correct ligation products (see above). The calculations of the volumes used in the ratios took into account that the pBS length was 1 kb and the inserts were ~0.5 kb. 5 controls were run i.e. plasmids that were cut but not dephosphorylated, cut and dephosphorylated, uncut, and negative controls containing no plasmid and no insert. Ligation was carried out using a 5x buffer at 10-15^{o}C overnight.

In all the stages above, the plasmid DNA was extracted with phenol/chloroform followed by alcohol precipitation.

4.2.3.4.3 Transformation

Transformation is an artificial process of introducing plasmid DNA into bacteria. The bacteria are treated with mixtures of divalent cations to temporarily increase their permeability to small DNA molecules. To identify transformants, selectable markers are used. These confer a new phenotype such as resistance to antibiotics. The phenotypic trait we made use of was resistance to ampicillin.

Competent *E. coli* XL1 (Stratagene®) bacterial cells were transformed by pBS (Stratagene®) using $MgCl_2$ / $CaCl_2$ followed by heat shock. The transformed cells were plated onto small selective ampicillin agar plates and incubated overnight (17-20 hr). Different cell titre plates of 0.5, 5 and 50 µl cells were used.

To identify bacterial colonies that contained recombinant plasmids, we used the α-complementation method by the addition of 40 µl X-Gal and 4 µl IPTG onto the surface of the agar plates. The vector used contains an *E.coli* gene (*LacZ*) that encodes for an amino end protein fragment of ß-galactosidase (146 amino acids). The host cell encodes for the carboxyl end of the protein. When these two

combine, α-complementation takes place and the enzyme becomes active. The colonies, growing in the presence of sugar X-gal (a galactoside), appear blue. Embedded in *LacZ* is a polyclonal site. Its presence only adds a few innocuous amino acids to the amino terminal protein fragment. If a foreign DNA is cloned into the vector, a smaller amino end fragment incapable of α-complementation results. Such colonies, with recombinant DNA appear white.

Transformation results were as follows:

1. No colonies - This was expected in unligated dephosphorylated plasmids (thus indicating successful dephosphorylation) and the negative controls. Lack of growth of colonies with ligated or uncut plasmids indicated a failure in the transformation and the process was repeated.

2. Blue colonies only - This was the expected result in the control containing uncut plasmid DNA. It also indicated recircularisation of plasmid DNA due failure of dephosphorylation and subsequent failure to ligate foreign DNA, thus dephosphorylation and ligation were repeated.

3. Mainly white colonies - This indicated successful ligation. Ligation was never 100 %, the highest being approximately 80 %.

4. Few colonies - This indicated a low efficiency of transformation and called for a retrial.

The vector:insert ratio which transformed colonies with the highest efficiency was repeated on a larger scale and plated onto large 20 x 20 ampicillin agar plates.

4.2.3.5 Southern blotting

The colonies were transferred to nylon Hybond N membranes (Amersham®) by Southern Blotting and hybridised with $(AC)_{23}$ probe end-labelled ($\gamma^{32}P$) dCTP by a kinase reaction (Pharmacia Ready To Go®). The membrane was then autoradiographed. Positive colonies indicating $(CA)_n$ repeats were identified by overlaying the autoradiogram onto the original agar plates. These were then picked and transferred for overnight culturing and rescreening.

4.2.3.6 Rescreening

2 µl of positive culture was added to 500 µl luria broth / ampicillin and incubated in microtitre plates overnight at 37 °C with shaking, plated out, transferred onto nylon membrane and probed as above. Positive colonies were identified. These colonies were now possible candidates for recombinant plasmids with $(CA)_n$

microsatellites. To these was added 25 % glycerol so that they could be stored at –70°C for future sequencing.

4.2.3.7 **Isolation of plasmid DNA** (Stephen *et al.* 1990)

This method isolates high quality plasmid DNA suitable for DNA sequencing (dideoxy chain termination method). 1.6ml of the overnight culture was decanted into a microfuge tube centrifuged and drained. The pellet was resuspended in 180 µl GTE solution then 360 µl 0.2 M NaOH / 1%, mixed then 270 µl of 3M potassium acetate (pH 4.8) was added and further mixed, centrifuged and the DNA was extracted using phenol chloroform, precipitated in alcohol resuspended in 20 µl of sterile TE. DNA was then ready for sequencing or restriction enzyme digestion. We also used Wizard® miniprep kit (Promega®) for isolation of plasmid DNA following the manufacturer's instructions.

4.2.3.8 **Insert length analysis**

The insert length was analysed by restriction enzyme digestion with *XbaI* followed by *EcoRI*. The digested plasmid DNA was the checked in 2 % agarose gel and their sizes estimated using 123 bp ladder (2 µl). The fragments ranged from 250-1300 bp long. Inserts of 750 bp were selected for sequencing.

4.2.4 Sequencing

Sequencing was done manually using the dideoxy chain termination method or automated sequencer (ABI PRISM 377).

Dideoxy sequencing depends upon base-specific termination of enzyme catalysed primer extension reactions. The enzyme we used was T7 DNA polymerase (Pharmacia®). Four separate reactions were performed, all containing primer, template and four deoxynucleotides but each including a different chain terminating dideoxynucleotide. Dideoxynucleotide analogues are 2'3'dideoxynucleoside 5'-triphosphates (ddNTP's) which lack the 3'-OH group necessary for DNA chain elongation. In each reaction, a mixture of fragments are generated each terminated with the particular dideoxynucleotide present. Each fragment then represents occurrence of the corresponding deoxynucleotide in the sequence. When the products of the four reactions are electrophoresed side by side, the sequence of nucleotide addition is then deduced from the sequence in which successively larger fragments occur in the four lanes. The fragments were detected by incorporation of (α-^{35}S)dATP. The bands were resolved by running in a 6 % denaturing polyacrylamide gel followed by autoradiography.

To resolve ambiguities, we also used automated sequencing (ABI 377 PRISM). In this case, the recombinant plasmids used were recovered only by Wizard® miniprep according to the manufacturer's protocol.

4.2.5 Primers

4.2.5.1 Primer design

After sequencing, 6 $(CA)_n$ repeats and 1 $(TA)_{16}$ microsatellites were identified. These were selected on the basis of having 15 or more dinucleotide repeats. Primers for selected microsatellites were designed for the flanking conserved regions using a computer software package (Primer version 0.5). Considerations for primer design are:

- Primer length - Generally synthesised in the range 18-30 bases, though it is possible to amplify low complexity DNA (e.g. plasmids or previously amplified DNA) with shorter primers. Our primers ranged between 19-22 bp (see Table 3.1)
- They should have similar G+C content.
- Minimum secondary structure (self-complementarity)
- Low complemetarity with each other, particularly in the 3' region.
- Similar melting temperatures.

4.2.5.2 Primer Synthesis

The primers were synthesised using an automated synthesiser (391 DNA synthesiser (PCR-MATE®) Applied Biosystems®). I followed the manufacturer's instructions for eluting the oligonucleotides after synthesis

4.2.6 Polymorphism

PCR (polymerase chain reaction) was used to amplify the microsatellite loci in 1-10ng of DNA from 14 unrelated ostriches (7 males and 7 females) for determination of heterozygosity and allele frequency. Ability to amplify ostrich egg membranes (farm source) was tested.

4.2.6.1 PCR amplification

PCR amplification reactions were carried out in a GRI Minicycler thermal cycler using DNA from 14 individuals (7 males and 7 females). Amplification reactants in a total volume of 25 µl were: 0.1 µl template DNA (about 1-10 ng), 2.5 µl 10x buffer (50 mM KCl, 10 mM Tris pH 9.0 (at 25 ^{o}C), 0.1 % triton X-100), 1.5 µl $MgCl_2$ (1.5 mM), 0.2 µl dNTPs (0.2 mM of each), 0.5 µl 12.5 pmol of each primer, 1.0 µl (0.5 U) dil. taq polymerase (0.1 µl taq(5U /

μl):0.9μl H_2O), 19.2 μl H_2O. 25 μl of mineral oil was used to seal off the reactants.

The amplification conditions were: initial denaturating 94 ^oC for 4 minutes followed by 35 cycles of: denaturation 94 ^oC for 10 seconds, annealing temperature 56-59 ^oC for 30 seconds depending on the primer used (Table 3.1), primer extension 72 ^oC for 30 seconds, and final extension of 72 ^oC for 5 minutes.

The PCR reactions had to be optimised to avoid multiple non-specific amplification, low yields of desired products (inadequate amplification) or lack of products (no amplification). This was done by adjusting the annealing temperature and concentrations of $MgCl_2$, dNTPs and DNA polymerase. Contamination of primers was encountered. In a few cases, the stocks were also contaminated necessitating the resynthesis of primers. A set of new Eppendorf® pipettes was purchased and isolated for use on PCR DNA free manipulations.

The PCR products were then resolved on a 6 % denaturing polyacrylamide gel vertical electrophoresis at 1100 V, 30-50 mA for 4 h. The PCR products were transeferred by Southern blotting onto a nylon membrane, hybridised with $(AC)_{23}$ end-labelled [γ-^{32}P]-dATP

and exposed to autoradiographic film (2-3 days) for visualisation (Cohen *et al.* 1992).

4.2.7 Ratite cross-amplification

The other ratite DNA tested foramplification was Darwin's rhea (*Pterocnemia pennata*), cassowary (*Casuarius casuarius*) and emu (*Dromaius Novaehollandiae*). The source of the DNA was feather and ancient skin DNA from the British Museum.

4.2.8 Other tissue extraction

Ostrich egg membranes

The ostrich egg membrane DNA was extracted by digestion with Proteinase K and the Phenol / chloroform method described earlier.

Ratite feathers (1 ostrich, 2 cassowary and 4 emu)

For the feathers, we used the method described by Taberlet & Bouvet (1991).

Extraction of DNA from Museum specimens.

DNAs from samples of skins from 4 museum specimens of cassowaries were extracted as described by Pääbo (1990).

The DNA was concentrated and purified by Centricon 30 microconcentrators according to the manufacturer's instructions

4.3 Results

25 positive clones were sequenced. Seven of these had both uninterrupted repeates of 15 or more dinucleotide repeats and suitable flanking regions for primers. Ten had less than 15 pairs of repeats and showed little or no polymorphism, two did not have suitable flanking regions and six had no repeat sequences or very dispersed repeats. Seven microsatellites were polymorphic with a mean allele number of 6.0 (range 4.0-9.0). The average observed heterozygosity was 0.58 (range 0.40-0.79)(Table 4.1). In every case, the microsatellite loci were amplified from DNA extracted from ostrich chick egg membranes collected at Maasai ostrich farm soon after hatching.

Four of the primers amplified loci in Darwin's rhea (*Pterocnemia pennata*), five in cassowary (*Casuarius casuarius*) and six in the emu (*Dromaius novaehollandiae*) (Table 4.2). Limited sample sizes meant that the extent of polymorphism was not estimated.

Table 4.1

Characterisation of seven microsatellite loci in *Struthio camelus massaicus*_Neumann. The number of alleles was obtained for 14 unrelated individuals. OSM1 was isolated by O. Hannote and J. Graves at the University of Leicester, UK. The accession nos. for OSM1-7 are AF003729-AF003735 (EMBL database)

Locus	length of PCR product	Primer sequence (5' - 3')	Repeat motif	Annealing temp.(^{O}C)	No. of alleles	H_o
OSM1	110	F: AATCTGCCTGCAAAGACCAG R: TCCCAGTCTTGAAGTCAGCA	$(CA)_{17}$	57	9	0.50
OSM2	121	F: AAGCCACGGCAATGAATAAG R: CCTCAACCATTCTGTGATTCTG	$(CA)_{22}$	57	6	0.71
OSM3	157	F: ATCTCCTTTGCTGGTGCAAT R: CCGGGGGGATTTCTTATGT	$(CA)_{15}$	57	4	0.56
OSM4	134	F: ATCACTTTGCTGAAGTCAAAGG R: CTAACAGAGATCTGGGCGGA	$(TA)_{16}$	56	5	0.53
OSM5	232	F: GTGGATCAGTTCAATCCTTGC R: GCCCAAGAAAATGATGGAGA	$(CA)_{20}$	59	6	0.57
OSM6	108	F: TTTGACCATTCAGCATGCAT R: AGAACTGCTGCCTTTCCTCA	$(CA)_{15}$	57	5	0.79
OSM7	215	F: AGCATACACATGCAGACCCC R: TGTTTCCTGCCATTCTGTCA	$(CA)_{16}CT(CA)_5CT(CA)_{25}$	58	7	0.40

Table 4.2.

Results of amplifications in other ratite species DNA screened using the seven ostrich microsatellites. N is number of individuals tested. The numbers in bracket indicate how many samples amplified. + indicate samples that amplified while - indicate those that did not. DNA was extracted from museum skin samples for the cassowaries (Accession numbers British Museum of Natural History (BMHN) reg. Nos. 1874.22, 11916.5.30.1481-1484, 1914.8.26.1) using the protocol of Pääbo 1990 and from plucked feather for emus following the protocol of Taberlet & Bouvet (1991).

Locus	Cassowary n=6	Rhea n=4	Emus n=4
OSM 1	+(2)	+(4)	+(4)
OSM 2	+(2) n=2	+(4)	+(4)
OSM 3	+(3)	+(3)	-
OSM 4	+(2)	-	+(4)
OSM 5	-	-	+(2)
OSM 6	-	-	+(4)
OSM 7	+(1)	+(2)	+(4)

4.4 Discussion

Seven ostrich microsatellites were isolated and scored for polymorphism. I used these as genetic markers to genotype and identify individuals in a population at the Nairobi National park. Genetic identification was then used to carry out parentage analyses and relatedness coefficient estimates. These measures enable

investigation of the development and maintenance of the unique ostrich communal nesting as discussed in the succeeding chapters.

The average observed heterozygosity was lower than those reported for other species of birds (Piertney & Dallas 1997, Primmer *et al* 1995, Neuman & Wetton 1996), but may be underestimates for wild ostriches since the samples were obtained from an Ostrich farm where there may have been some inbreeding.

The microsatellite loci amplified ostrich chick egg membranes collected from Maasai ostrich farm soon after hatching. We made use of this ability to genotype embryonic membranes from developing eggs collected at Nairobi National Park for parentage analysis as discussed in chapter 5.

The primer sequences were all found to be conserved in at least some of the other ratites examined. Although the split between the rheas and the ostrich is estimated to have been 80 Mya (Cracraft 1974, Sibley & Ahlquist 1990), four of the primers amplified loci in Darwin's rhea (*Pterocnemia pennata*) (Table 3.2). The split between the ostrich and the cassowary (*Casuarius casuarius*) and emu (*Dromaius novaehollandiae*) may be even more ancient (Sibley & Ahlquist 1990, Diamond 1983), but five and six loci were amplified respectively.

Chapter 5

REPRODUCTIVE CONSEQUENCES OF THE OSTRICH *Struthio camelus massaicus* COMMUNAL NESTING SYSTEM.

5.1 Introduction

The ostrich has a unique breeding system. Its communal nesting system consists of a major female and several minor females laying in one nest. Parental care such as nest guarding, incubation and escorting the chicks is exclusively carried out by the major female and the territorial male. The minor females make no contribution to the nest beyond laying eggs in it. The territorial male however, mates with these minor females within his territory. Therefore the nest contains eggs that the major female has not laid and the male may not have fertilised. The major female and territorial male may be investing in chicks that are not their offspring. How does the pair avoid this?

Here, I estimate the reproductive success of the individuals involved in this complex breeding system i.e the territorial male, major female and the minor females. I used eight polymorphic microsatellite markers to genotype the central clutch eggs' embryos of four neighbouring nests and their parents at the Nairobi National

Park. All the males analysed were nesting territorial males. Six females were analysed: the four major females and two other major females of nests not included in the analysis.

Three predictions to be tested are:

1. **Major females are able to ensure that their eggs are incubated.**

Bertram (1982), using qualitative measures such as shape, size, pore size and pattern, came to the conclusion that the major females selected her eggs for retention and incubation in the central clutch.

2. **The major females' eggs are fertilised by the territorial males.**

The territorial male and the major female form a pair bond that mate frequently, guard, incubate the eggs and broods and eventually escort the chicks (Hurxthal 1979, Bertram 1992).

3. The territorial males fertilise some, but not all, of the other females' eggs in the nest.

The breeding females range over a large area covering the territories of 4-7 males. The females mate with these territorial males (Hurxthal 1979). The territorial male has also been observed to mate preferentially with new females that enter their territories (Hurxthal 1979).

5.2 Materials and Methods

5.2.1 Samples

Eggs from five abandoned nests were collected from Nairobi National Park (Table 3.1). Embryonic tissues, mainly chorio-allantoic egg membranes, were collected from each of the fertile central clutch eggs. Nest 5 was excluded as all the eggs from this nest were undeveloped and no embryonic tissues could be found. A total of 25 adults and 61 chicks were sampled. Eight of the adults were territorial male and major female pairs while the other seventeen were two other major females and fifteen additional adults. These individuals, presumably unrelated, hatched from eggs collected within the southern migratory area of the Park by Maasai Ostrich Farm, with the permission of Kenya Wildlife Services, as start-up stock. Feather samples were also collected from a family of two males, four females and nine offspring at Maasai Ostrich Farm. This was the only family whose record was available and whose chicks had recently hatched. At Nairobi National Park, I initially set out to measure reproductive success by collecting feather samples from hatched chicks. However, since all the nests were destroyed, this was not possible. I estimated parentage by assigning the number of eggs that contained developing chicks fathered or mothered by interacting individuals. I will

therefore refer to reproductive output rather than hatching success. All samples collected were frozen at -20°C until analysed.

5.2.2 Loci typing

Genomic DNA was extracted as described in chapter 3. Eight highly polymorphic microsatellite loci were used for the Nairobi National Park population and five on the Maasai Ostrich Farm family. Those used on the NNP population were OSM1, OSM2, OSM4, OSM5, OSM6, OSM7 (Kimwele *et al*, 1998), List005 and List009 (Kumari & Kemp, 1998). OSM3 was excluded from the analysis as only three alleles were scorable, and despite four repeated attempts, failed to amplify two nests (4 and 7) and male nest 3. OSM1, OSM2, OSM3, OSM4 and OSM7 were used on the Maasai Ostrich farm family. Amplification of the loci was carried out by polymerase chain reaction (PCR). The cycling conditions were as in Kimwele *et al* (1998) and Kumari & Kemp (1998) with the exception that the number of cycles was reduced to 25 to improve resolution by limiting allele slippage. The PCR products were resolved in 6% denaturing polyacrylamide (PAGE) gels as described in chapter 3. The alleles were then typed according to size using a 10bp ladder

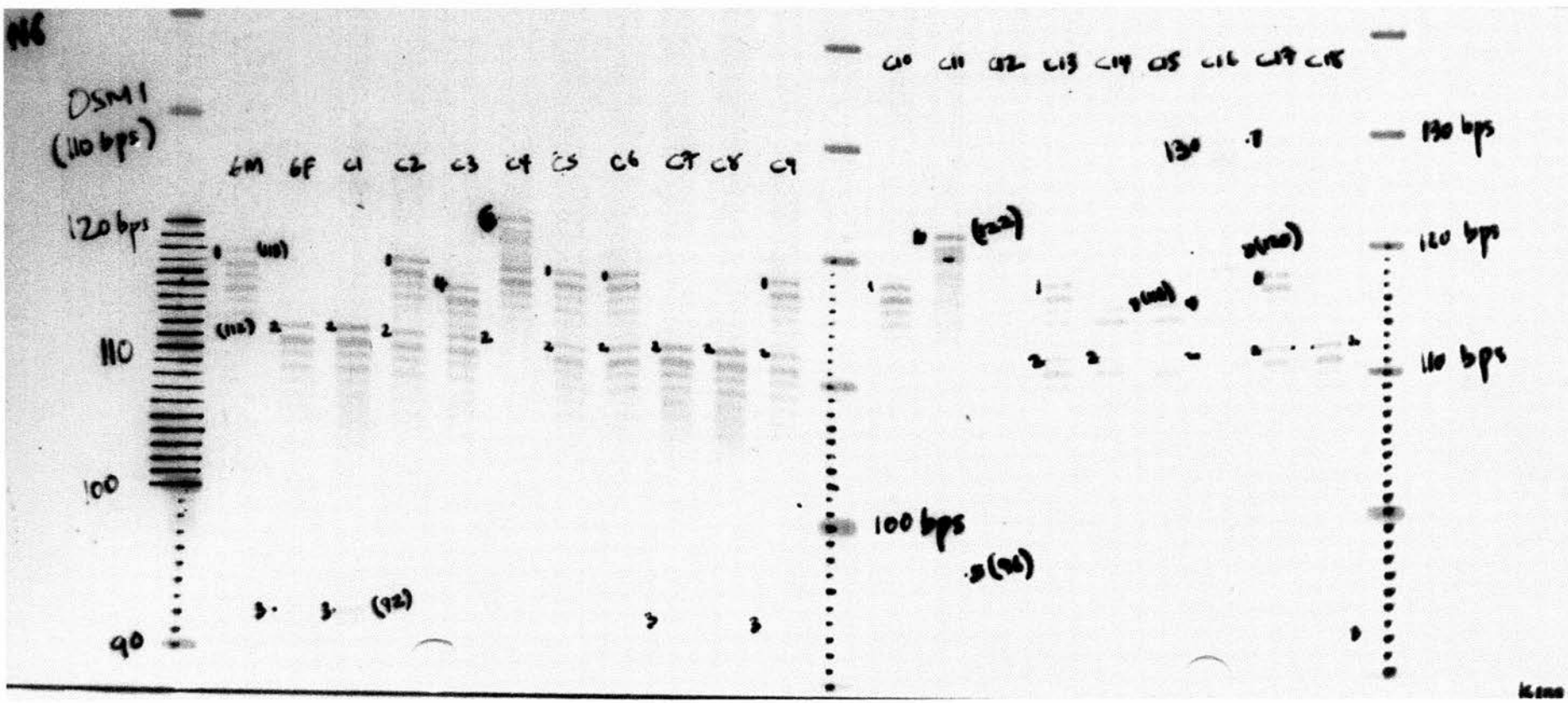


Figure 5.1 Nairobi National Park family 6 genotyped woth OSM1 microsatellite locus. From left to right; 10 bp ladder, territorial male, major female, eggs 1-9, 10 bp ladder, eggs 10-18, 10 bp ladder. The alleles ranged between 110 and 130 bp.

(Promega®) across the eight loci to give genotypic profiles of the individuals (Figure 5.1). A total of 25 adults and 61 chicks was genotyped.

5.2.3 Paternity inference

I used a likelihood based method for parentage inference, the *Cervus* program (Marshall *et al* 1998). This program defines a statistic Δ for resolving parentage by running a simulation using allele frequencies from the study population in question. This is used to generate criteria for Δ that permit assignment of paternity to the most likely male with a known level of statistical confidence.

Likelihood analysis, using data as a starting point, evaluates hypotheses (H_1, H_2). Using these data the likelihood (L) of one hypothesis (H_1) evaluated relative to another (H_2) is found as

$$L(H_1,H_2|\,D) = \frac{P(D\,|H_1)}{P(D\,|H_2)} \qquad (1)$$

Where $P(D|H_i)$ is the probability of obtaining data D under hypothesis H_i. Data D are the genotypes of offspring, mother and alleged father. If the mother's genotype is unknown, D is the

offspring's and alleged parent's (mother or father) genotypes. The hypothesis of interest H_1 is that the alleged parent is the true parent, and this is tested against the hypothesis H_2 that the alleged parent is an unrelated individual selected at random from the population. In cases where the mother's genotype is unknown, as was the case here, the likelihood ratio is:

$$L(H_1,H_2|g_a,g_o) = \frac{T(g_o|g_a).P(g_a)}{P(g_o).P(g_a)} = \frac{T(g_o|g_a)}{P(g_o)} \quad (2)$$

Where $P(g_o)$ is the frequency of the offspring's genotype

$P(g_a)$ is the frequency of the alleged parent's genotype

$T(g_o|g_a)$, the Mendelian segregation or transition probability is the probability of the offspring's genotype given the genotype of the alleged parent (Marshall *et al* 1998).

Allele frequencies may only be used to estimate genotype frequencies if Hardy-Weinberg equilibrium holds. When several unlinked marker loci are used in parentage inference, the likelihood ratios derived at each locus are multiplied together and the natural ($\log_e$) logarithm taken, this is termed the LOD (Meagher, 1986). A LOD score of zero implies that the alleged father is equally as likely to be the father of the offspring as a randomly selected male. A

positive LOD score implies that the alleged father is more likely to be the father of the offspring than a randomly selected father (Marshall *et al*, 1998).

Using the likelihood approach eliminates exclusion of parentage on the basis of rare allelic mismatches. An allelic mismatch may reflect true parentage exclusion. However, it may also arise from erroneous laboratory typing, the presence of null alleles or mutations leading to erroneous exclusion. Also, if several individuals are not excluded, the likelihood method can be used to award parentage to the most likely parent. This method is also useful in awarding paternity to unsampled individuals, e.g. when parentage cannot be awarded to any of the individuals sampled.

Paternity assignment using LOD scores

The Δ statistic is used to discriminate between individuals. This statistic is the difference between the most likely parent and the second most likely parent. Only LOD scores of greater than zero are considered. If n is the number of candidate parents, and the LOD score of parent i is denoted by LOD_i and the parents are ranked such that $LOD_i \geq LOD_{i+1}$ (i.e the most likely male is denoted LOD_1), then Δ is defined as follows:

$n \geq 2$, $\Delta = LOD_1 - LOD_2$

$n = 1$, $\Delta = LOD_1$

$n = 0$, Δ undefined

Where n = number of candidate parents whose LOD score >0

Without a threshold LOD score of zero, Δ is sensitive to LOD_2. If LOD_2 is very negative (typically when all candidate parents except the most likely mismatch the offspring at several loci), Δ is large whatever the value of LOD_1. A threshold LOD score stabilises Δ because Δ always lies between zero and LOD_1.

Simulation of paternity inference

Simulations are used to assess the significance of Δ values. *Cervus* simulation analysis emulates the steps of paternity inference using allele frequency at loci screened in a given study population. Parallel simulations are carried out with or without maternal genetic data. Assuming a Hardy-Weinberg equilibrium and using the population allele frequencies, parental and candidate parents genotypes are generated and, by Mendelian sampling, offspring genotypes. A total of 10,000 tests are usually sufficient to generate distributions of Δ. Adjustments are made to reflect unsampled

candidates, missing loci and incorrectly typed loci. In the analysis, I assumed that there were 10 candidate parents. This is more than the number of possible fathers estimated in field studies. Hurxthal (1979) estimated that 4-7 candidate males copulated with a female and up to 11 females laid in a nest. Using the marker set developed for testing a red deer population (three protein (two alleles) and nine microsatellite (6-13 alleles)), Marshall (1998) showed that if the number of candidate individuals was far greater than 10 and the mother was unknown, parentage could only be assigned at 80% confidence. Using eight highly polymorphic microsatellite makers, I was able to achieve an exclusion probability of 0.997 and 95% confidence in assigning paternity in the absence of known mothers. Missing loci were estimated from the genetic data. Typing error (this includes mutations and null alleles) can be assessed from a known pedigree. As in this case, natural populations offer little opportunities for such pedigree analysis and none exists for my study. A high frequency of null alleles would be expected to result in a homozygote excess however, our population, across all loci, was in Hardy-Weinberg equilibrium (Table 5.1). I therefore had to rely on Marshall's estimated red deer error rate of 0.01, also based on micrasatellite data (1998). As the error rate rises, the percentage of paternity resolution for a given confidence level falls.

LOD scores are then calculated and the most likely and second most likely individuals are used to calculate Δ along with its significance. To find the critical values of Δ the program compares the distribution of Δ scores for cases where the most likely male was the true father with those where the most likely male was not the true father. If a 95% confidence criterion is set, the program identifies the value of Δ such that 95% of Δ scores exceeding this value are obtained by the true fathers. If the program fails to find such a value (typically because the resolving power of the markers is insufficient), the critical value is set to an arbitrarily high value of 99.99. Our markers were able to resolve parentage at 95% confidence in all cases where resolution was possible.

5.2.4 Hardy-Weinberg test

The program *Genepop ver3.1d* (Raymond & Rousset, 1995) was used to compute exact tests for Hardy-Weinberg equilibrium among pairs of loci by two estimates of F_{is}, Weir & Cockerhams (W&C) (1984) and Robertson & Hill (R&H)(1984). This program uses a Markov chain method to estimate without bias the exact probability of this test (Guo & Thompson 1992). The null hypothesis is that there is random union of gametes. The population analysed

across loci was in Hardy-Weinberg equilibrium (Table 5.1). This indicated that there was no population substructure, no selection acting on any of the loci and no bias towards typing of any genotypes. There were also no (or very low levels of) null alleles segregating in the population and no locus segregating in a sex chromosome. Population substructure is likely to lead to deviations from Hardy-Weinberg equilibrium at all loci, whereas other causes of deviation are usually locus specific.

Table 5.1 Hardy Weinberg probability test, across eight loci, of 25 adult individuals from the Nairobi National Park ostrich population (Appendix 1; N6male to F8).

			Fis	Fis
Loci	P-value	S.E	Weir & Cockerhams	Robertson & Hill
1	.1583	.0138	-.121	-.071
2	.5138	.0133	+.120	+.075
3	.6064	.0080	+.127	+.035
4	.7861	.0145	-.032	-.023
5	.9927	.0009	-.022	-.026
6	.0880	.0066	+.061	+.096
7	.0607	.0079	+.274	+.280
8	.3661	.0278	+.038	+.031

All (Fisher's exact probability method): $\chi^2_{(16)}$=19.0 P=0.2692

5.2.5 Linkage disequilibrium test

The markers were tested for linkage disequilibrium .The null hypothesis (Ho) was that the genotype at one locus was independent of genotypes at the other loci. *Genepop* creates a genotype combination contingency table for all pairs of loci in the population and then performs a probability test using a Markov chain. The markers used were not in linkage disequilibrium (Table 5.2).

Table 5.2 Chi-square values of genotypic linkage disequilibrium analysis of eight loci within the ostrich population (n=25). There was no significant linkage disequilibrium between any interacting pairs of loci (df=2).

Locus	OSM1 1	OSM2 2	OSM4 3	OSM5 4	OSM6 5	OSM7 6	List005
OSM1	-						
OSM2	0.00	-					
OSM4	0.00	0.00	-				
OSM5	0.00	2.38	1.85	-			
OSM6	4.63	0.00	0.85	0.37	-		
OSM7	0.81	0.00	0.00	0.54	1.00	-	
List005	0.72	0.00	0.00	0.79	1.52	0.00	-
List009	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Definition of reproductive output terms

Both the territorial male and major female had extra-pair eggs fertilised by other mates. These were estimated as the number of eggs fertilised and laid in a central clutch, but not by either the territorial male and the major female.

1. EPP(I) - refers to intra nest extra pair paternity i.e eggs of other females' fertilised by him and laid in his nest.
2. EPP(E) - refers to extra nest extra pair paternity, eggs of other female fertilised by him and laid in nests of other territorial males
3. EPP - refers to the sum of EPP(I) and EPP(E): the total number of incubated eggs fertilised by the territorial male but not laid by the major female across all the tested nests.
4. EPM(I) - refers to intra nest extra pair maternity, eggs laid by the major female in her own nest but fertilised by males other than the territorial male.
5. EPM(E) - refers to extra nest extra pair maternity, eggs laid by the major female in other females' nests and fertilised by males other than the territorial male.
6. EPM refers to the sum of EPM(I) and EPM(E), reflecting the total number eggs laid by the major female but not fertilised by the resident territorial male, across all nests.

7. IPP – refers to Intra pair parentage, eggs laid by the major female and fertilised by the territorial male.

5.3 Results

5.3.1 Reproductive output

A total of 61 eggs from 4 nests was analysed. Ten candidate parents including four pairs of territorial male and major female at a nest were sampled. We could not assign fathers to twelve and mothers to six eggs at either 95% or 80% confidence levels (Tables 5.3-5.10). Only two eggs were unassigned to both mother and father (Table 5.15). Thus a maximum of twelve fathers and six mothers were unsampled. Since survival was zero, wherever reference to reproductive success (or offspring) is made, this should be understood as an estimate of reproductive output essentially representing the number of eggs fertilised and undergoing embryonic development.

5.3.1.1 Male reproductive output

Parentage inference revealed a very high incidence of male extra-pair fertilisations (EPF's) across all nests. Out of 61 eggs genotyped, only 17 (27.9%) were successfully fertilised within the

pair bond of territorial male and major female. There were 32 extra-pair fertilisations representing a high EPF of 52.5%. Males incubating nests 3, 4, 6 and 7 fertilised 9, 8, 8 and 10 eggs respectively in their clutch representing 50.0, 61.5, 44.4 and 83.3 % of the incubated eggs respectively (Figure 5.2). Other territorial males gained mean fertilisations of 3, 2.5, 2.5 and 2 eggs in nests 3, 4, 6 and 7 respectively representing 16.7, 19.2, 13.9 and 16.7% respectively. This mean fertilisation success by other males was an overestimate as the assumption was that only a single male was unsampled (Tables 5.4, 5.6, 5.8 and 5.10) and that any male who copulated with the females achieved some fertilisation success as detected by our markers. Males who mated with a female but did not fertilise her eggs could not be detected therefore the actual fertilisation success variance may have been underestimated and the mean success of other territorial males was overestimated since in reality, more males would be expected to have mated with the females.

In nest 3, the resident territorial male fertilised 9 of the 18 incubated eggs, 2 belonging to the nest's major female and 7 to 4 other females. He also fertilised 4 other eggs incubated in nest 6, 3 with nest 6 major female and 1 with nest 8 female.

Nest 4 resident territorial male fertilised 8 of the 13 eggs incubated in his nest, 3 belonging to his major female and 5 laid by other minor females. He had 2 eggs, that he fertilised but were laid outside his nest – with Nest 8 female and laid in nest 3, and with his own female but laid in nest 6. This was the only case of an egg fertilised within a pair but laid outside the pair's nest.

In nest 6 out of the 18 eggs incubated in the central clutch, the resident territorial male fertilised 8, 4 belonging to his major female and 4 with other females. He also fertilised 4 other eggs that were laid outside his nest and all laid in nest 4, 1 by Nest 4 female and 3 by Nest 3 female.

In nest 7 the resident territorial male fertilised a total of 10 eggs out of the 12 that he incubated. 7 of these were with his major female and 3 were with other minor females. He also fertilised 4 other eggs outside his nest, 3 in nest 3 one each belonging to Nest 2 female, Nest 6 female and an unsampled female and 1 in nest 6 belonging to nest 6 female.

One to a maximum of seven (assuming each unsampled male to be a different male) other males fertilised 9, 3, 4 and 1 (Tables 5.4, 5.6, 5.8 and 5.10) minor females' eggs respectively reflecting the incidence of intra brood parasitism (IPB). The resident male gained EPP(I) of, on average, 57.8% of eggs laid by minor females in his

nest (43.8, 62.5, 50.0, and 75% respectively). The total extra-pair fertilisations (EPP) gained by the sampled individual territorial males was 11, 6, 8 and 7 a mean of 8.0 ± 2.16(SD), for males 3, 4, 6 and 7 representing 84.6, 60.0, 66.7 and 50.0% of their total fertilisation success of 13, 10, 12 and 14 eggs respectively. Across the nests there was no difference between number of eggs fertilised by the resident territorial male that were laid by the major female (IPP), laid by other minor females in his clutch (EPP(I) and laid by minor females in other territorial male nests (EPP(E)) (ANOVA $F_{2,11}$=0.74 P=0.50).

Assuming random central clutch egg fertilisation by the males that fertilised a given nest, and assuming that only one male was unsampled, then the expected number of eggs fertilised per male would be five in nest 3, four in nest 4, four in nest 6 and six in nest 7, a mean of 4.75 ± 0.96. However, resident territorial males had higher total fertilisation success across all nests of 9, 9, 8 and 10 for nests 3, 4, 6 and 7 males respectively, a mean of 9.0 ± 0.8 (SD). This was not a significant difference ($\chi^2_{(3)}$=3.80 P=0.28).

Across all nests, there was a total of 17 of within pair parentage eggs, 32 extra pair eggs and 17 eggs resulting from intra specific brood parasitism. Overall fertilisations across all nests revealed that resident males fertilised 4.25 ± 2.06 (SD) of their major

female's eggs (IPP) and 8.0 ± 2.16 (SD) eggs laid by minor females (EPP). This difference was not significant (t=1.94 df=3, P=0.15). The resident males fertilised as many minor females' eggs on their nests, 4.75±1.71 (SD) as did other males combined, 4.25±3.40 (SD) (t-test t=0.52 P=0.64).

Table 5.3 Nest 3 Male Parentage assignment within the territorial nest. The mother was unsampled. For this and subsequent tables, * denotes 95% confidence and + denotes 80% confidence

Offspring ID	Offspring loci typed	Prob. non-exclusion	Candidate parent ID	CP loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N3-c1	7	8.56E-05	N3male	7	7	9.20E-01	5.72E-01	*
N3-c2	7	2.49E-03	N3male	7	7	2.33E-01	2.33E-01	*
N3-c3	7	4.59E-03	N7male	8	7	1.41E+00	2.39E-01	*
N3-c4	7	2.09E-03	N3male	7	7	-1.36E-01	0.00E+00	
N3-c5	6	3.27E-04	N4male	8	6	4.53E-01	4.53E-01	*
N3-c6	7	1.90E-03	N7male	8	7	7.60E-01	7.60E-01	*
N3-c7	7	3.84E-03	N3male	7	7	-7.58E-01	0.00E+00	
N3-c8	7	5.96E-03	N3male	7	7	2.42E+00	2.29E+00	*
N3-c9	7	1.44E-03	N3male	7	7	7.57E-01	1.49E-01	*
N3-c10	7	1.22E-03	N6male	7	6	-1.53E+00	0.00E+00	
N3-c11	7	5.88E-03	N4male	8	7	-3.25E-03	0.00E+00	
N3-c12	7	4.18E-03	N3male	7	7	5.16E-01	5.16E-01	*
N3-c13	7	9.93E-03	N7male	8	7	9.31E-01	9.31E-01	*
N3-c14	7	7.67E-05	N3male	7	7	1.45E+00	1.03E+00	*
N3-c15	6	9.16E-03	N3male	7	6	5.88E-01	5.88E-01	*
N3-c16	7	1.38E-03	N3male	7	7	-2.91E-01	0.00E+00	
N3-c17	7	2.37E-03	N3male	7	7	1.36E+00	1.36E+00	*
N3-c18	7	2.37E-03	N3male	7	7	1.36E+00	1.36E+00	*

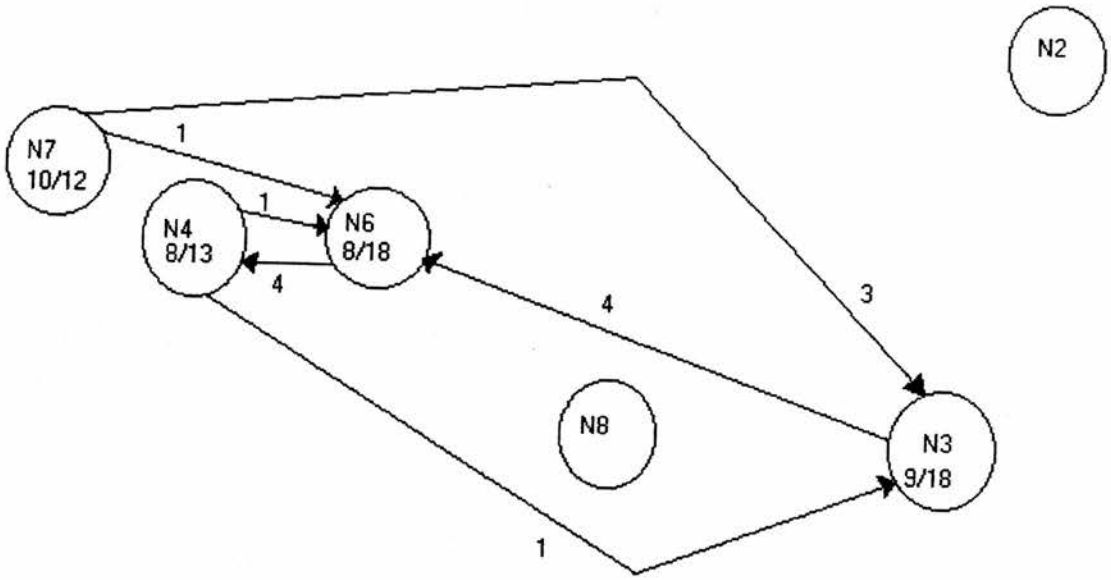


Figure 5.2 Territorial male reproductive output in their own and other males' nests. The arrows indicate that a territorial male fertilised eggs in another territorial male's nest. The numbers indicate the number of eggs that such a male fertilised. The encircled numbers indicate the proportion of eggs a territorial male fertilised in his nest.

Table 5.4 Nest 4 Male parentage assignment within his territorial nest. The mother was unsampled.

Offspring ID	Offspring loci typed	Prob. non-exclusion	Candidate parent ID	CP loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N4-c1	8	8.32E-04	N4male	8	8	1.23E+00	1.23E+00	*
N4-c2	8	1.19E-03	N4male	8	8	3.32E+00	3.13E+00	*
N4-c3	8	1.53E-03	N4male	8	8	8.36E-01	8.36E-01	*
N4-c4	8	5.83E-04	N4male	8	8	2.00E-02	2.00E-02	*
N4-c5	8	3.73E-04	N4male	8	8	4.63E+00	4.63E+00	*
N4-c6	8	1.10E-03	N4male	8	8	5.32E+00	5.32E+00	*
N4-c7	8	8.20E-04	N4male	8	8	-9.29E-02	0.00E+00	
N4-c8	8	1.79E-04	N6male	7	7	2.48E+00	2.48E+00	*
N4-c9	8	1.38E-03	N6male	7	7	2.34E+00	2.34E+00	*
N4-c10	8	1.11E-03	N6male	7	7	2.22E+00	1.82E+00	*
N4-c11	8	1.11E-03	N6male	7	7	2.22E+00	1.82E+00	*
N4-c12	8	2.23E-04	N4male	8	8	1.63E+00	1.63E+00	*
N4-c13	8	1.43E-03	N4male	8	8	3.66E-01	3.66E-01	*

Table 5.5 Nest 6 male parentage assignment within his territorial nest. The mother was unsampled.

Offspring ID	O loci typed	Prob. non-exclusion	Candidate parent ID	Candidate Parent loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N6-c1	7	1.19E-02	N7male	8	7	-5.65E-01	0.00E+00	
N6-c2	7	2.52E-03	N6male	7	7	2.32E+00	1.15E+00	*
N6-c3	7	3.19E-03	N7male	8	7	9.38E-01	1.87E-01	*
N6-c4	7	7.25E-04	N6male	7	7	-3.94E-01	0.00E+00	
N6-c5	7	4.38E-03	N6male	7	7	1.21E+00	5.90E-01	*
N6-c6	7	5.95E-03	N6male	7	7	2.07E+00	4.21E-01	*
N6-c7	6	2.88E-02	N3male	7	5	1.23E+00	1.23E+00	*
N6-c8	7	7.80E-03	N3male	7	6	1.84E+00	1.84E+00	*
N6-c9	6	2.55E-02	N6male	7	6	4.72E+00	4.72E+00	*
N6-c10	7	5.80E-04	N3male	7	6	5.73E-01	5.73E-01	*
N6-c11	7	2.64E-04	N3male	7	6	-8.49E-01	0.00E+00	
N6-c12	6	2.74E-02	N4male	8	6	8.24E-01	6.40E-01	*
N6-c13	7	5.26E-03	N6male	7	7	2.24E+00	8.06E-02	*
N6-c14	7	2.06E-02	N6male	7	7	3.12E+00	3.12E+00	*
N6-c15	7	1.14E-02	N6male	7	7	2.21E+00	2.21E+00	*
N6-c16	7	4.67E-04	N3male	7	6	1.51E+00	1.51E+00	*
N6-c17	7	3.33E-03	N6male	7	7	1.92E+00	1.92E+00	*
N6-c18	7	1.64E-03	N4male	8	7	-3.32E-01	0.00E+00	

Table 5.6 Nest 7 Male parentage assignment within his territorial nest. The mother was unsampled

Offspring ID	O loci typed	Prob. non-exclusion	Candidate parent ID	Candidate Parent loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N7-c1	8	7.51E-04	N7male	8	8	5.61E+00	5.61E+00	*
N7-c2	8	5.38E-05	N7male	8	8	-2.43E-01	0.00E+00	
N7-c3	8	3.04E-05	N7male	8	8	1.48E+00	1.48E+00	*
N7-c4	8	1.23E-04	N7male	8	8	2.31E+00	2.31E+00	*
N7-c5	8	1.60E-05	N7male	8	8	2.65E+00	2.65E+00	*
N7-c6	8	3.20E-05	N7male	8	8	-1.28E-01	0.00E+00	
N7-c7	8	2.43E-04	N7male	8	8	2.76E+00	2.76E+00	*
N7-c8	8	2.06E-04	N7male	8	8	4.92E+00	4.92E+00	*
N7-c9	8	9.29E-05	N7male	8	8	2.92E+00	2.92E+00	*
N7-c10	7	2.64E-05	N7male	8	7	2.74E-01	2.74E-01	*
N7-c11	8	6.62E-05	N7male	8	8	7.70E-01	7.70E-01	*
N7-c12	8	4.11E-04	N7male	8	8	4.21E+00	4.21E+00	*

5.3.1.2 Female reproductive output

The nesting major females of nests 3, 4, 6 and 7 had total reproductive output across all nests of 10, 7, 15 and 10 fertilised eggs respectively, a mean of 10.5 ± 3.32 (SD). Some of these eggs, laid as minor females in other major females' nests, were usually fertilised by other males (EPM(E)) other than the one they became a major female with, while others were laid in the major female's own nest fertilised either by the resident territorial male (IPP) or by other

territorial males (EPM(I). The total number of fertilised eggs laid by the major female showed less variation than exhibited by the minor or major strategy independently. Nest 3, Nest 4, Nest 6 and Nest 7 females incubated 2 (11.1%), 5 (38.5%), 10 (55.6%) and 8 (66.7%) of their own eggs in their nests respectively, a mean of 6.25 ± 3.50(SD). As minor females laying in various other nests, they laid 8, 2, 5 and 2 eggs respectively, a mean of 4.25 ± 2.87(SD). Though individual females had different success rate of incubating their own eggs, i.e. major female strategy (range 2-10) or having their own eggs incubated by other female i.e minor strategy (range 2-8), the total reproductive output across all nests was less variable (range 7-15) (Figure 5.3).

EPM(I), the number of eggs that were fertilised by males other than the resident male and incubated by the major female in her own nest was 0, 2, 6 and 1 respectively, a mean of 2.25 ± 2.63(SD). IPP, the number of eggs fertilised by the resident male and laid by the major female was 2 for Nest 3 female, 4 for Nest 4 female, 4 for nest 6 female and 7 for Nest 7 female, a mean of 4.25± 2.06(SD). All the IPP eggs were laid and incubated in the pair's nest with the exception of one egg laid by Nest 4 female and incubated in nest 6 (Tables 5.7, 5.8, 5.9 and 5.10). There was no significant difference between IPP,

EPM(I) and EPM(E) (ANOVA $F=_{2,9}$ 0.67 P=0.53). Although all the females employed both strategies, the major female strategy (IPP + EPM(I)), with a mean of 6.50±3.42(SD) did not produce a significantly greater number of incubated eggs compared with those laid as a minor female (4.0 ± 3.16(SD)) (t test, t=0.88, P=0.44, df=3). The non-significance may have been due to the high variance of both strategies together with a small sample size. This variance was mainly attributed to nest 3 female who had exceptionally low reproductive output in her nest, incubating just 2 eggs of her own. Conversely she had an unusually large number of EPM(E) eggs incubated; five in nest 4 and three in nest 6.

In a clutch of 20 eggs, the major female laid and incubated an estimated 7-11 eggs (Hurxthal 1979, Bertram 1982). Assuming the major female lay a total of 10 eggs, and that the distribution into central and peripheral clutchs, infertility and breakages were random, then the expected totals of incubated major females' eggs would be 5, 4, 4 and 7 against an observed 2, 5, 10, and 8 assigned by parentage analysis to nests 3, 4, 6 and 7 respectively. The observed number of centrally incubated eggs laid by the major female was significantly different from those expected if the eggs were randomly selected for incubation from the entire laid clutch ($\chi^2_{(4)}$=10.25 P=0.02).

Across all nests, extra pair fertilisations were evident in both sexes (Table 5.12). Major females had a mean of 6.25 ± 4.19(SD) eggs not fertilised by the resident territorial male (EPM). This was not significantly different from the mean number of eggs fertilised by the resident territorial male (IPP) of 4.25 ± 2.06 (SD) (t test, t=0.75, df 3, P=0.51).

The territorial males had a total reproductive success, across all nests, of 12.25±1.71(SD) while the major females' was 10.50 ± 3.32(SD). There was no significant difference between the success rate of the males and females (t test, t=1.09, df=3, P=0.35).

Table 5.7 Nest 3 Female Parentage assignment within her nest.

Offspring ID	Offspring loci typed	Prob. non-exclusion	Candidate parent ID	Candidate Parent loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N3-c1	7	8.14E-05	N6fem	7	6	2.76E-01	6.78E-03	*
N3-c2	7	2.40E-03	N3fem	7	7	2.89E+00	6.31E-01	*
N3-c3	7	4.32E-03	N2f	8	7	1.07E+00	1.07E+00	*
N3-c4	7	1.96E-03	N6fem	7	6	1.44E+00	4.22E-01	*
N3-c5	6	3.17E-04	F8	8	6	2.26E-01	2.26E-01	*
N3-c6	7	1.84E-03	N2f	8	7	-3.06E-01	0.00E+00	
N3-c7	7	3.90E-03	F8	8	7	1.44E+00	4.65E-01	*
N3-c8	7	5.39E-03	N6fem	7	6	8.81E-01	8.81E-01	*
N3-c9	7	1.35E-03	F8	8	7	-2.51E-01	0.00E+00	
N3-c10	7	1.25E-03	F8	8	7	1.20E+00	7.67E-01	*
N3-c11	7	5.78E-03	N2f	8	7	1.02E+00	8.25E-01	*
N3-c12	7	3.68E-03	F8	8	7	1.75E-01	1.75E-01	*
N3-c13	7	9.75E-03	N6fem	7	6	1.01E+00	1.01E+00	*
N3-c14	7	7.51E-05	F8	8	7	2.20E-01	2.20E-01	*
N3-c15	6	8.66E-03	N3fem	7	6	1.20E+00	1.99E-01	*
N3-c16	7	1.31E-03	F8	8	7	1.25E+00	1.25E+00	*
N3-c17	7	2.12E-03	N7fem	8	7	2.44E-01	2.44E-01	*
N3-c18	7	2.12E-03	N7fem	8	7	2.44E-01	2.44E-01	*

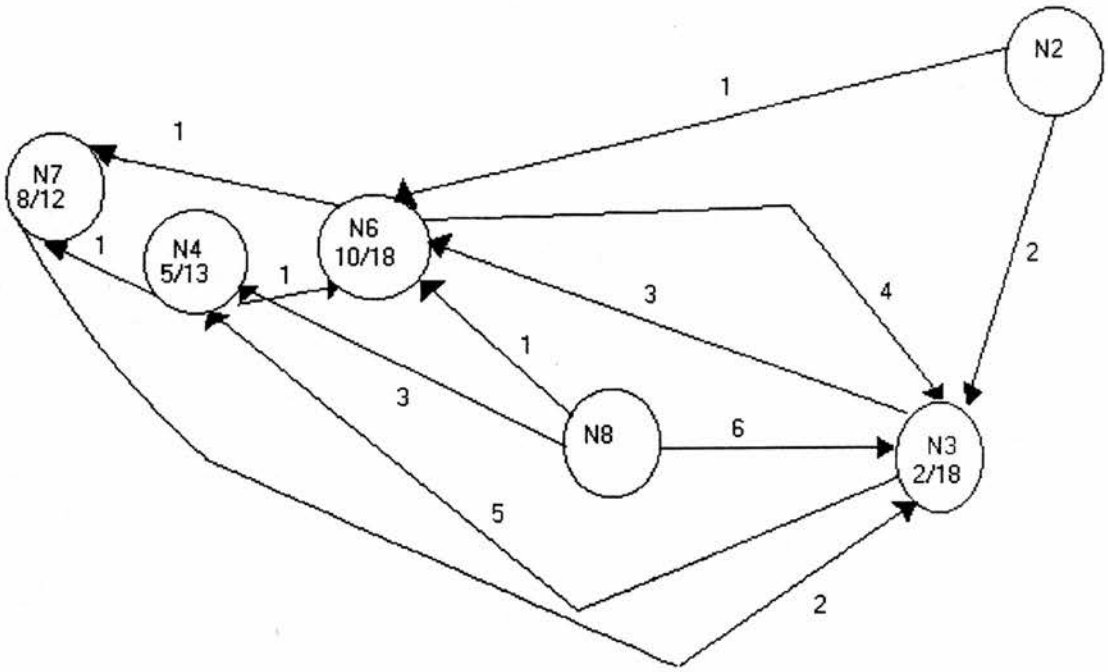


Figure 5.3 Laying patterns of identified major females at Nairobi National Park. An arrow indicates a major female laying in another major female's nest. The numbers encircled indicate proportion of eggs a major female contributed in her nest. The figures along the arrow indicate the number of eggs she laid in another female's nest

Table 5.8 Nest 4 female parentage assignment within her nest.

Offspring ID	Offspring loci typed	Prob. non-exclusion	Candidate parent ID	CP loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N4-c1	8	8.14E-04	F8	8	8	1.31E+00	1.31E+00	*
N4-c2	8	1.13E-03	N4fem	8	8	3.38E+00	3.38E+00	*
N4-c3	8	1.52E-03	N3fem	7	7	3.59E-01	2.37E-01	*
N4-c4	8	5.91E-04	N4fem	8	8	8.73E-01	6.57E-01	*
N4-c5	8	3.52E-04	F8	8	8	7.64E-01	7.64E-01	*
N4-c6	8	1.08E-03	N4fem	8	8	1.91E+00	1.91E+00	*
N4-c7	8	8.32E-04	N4fem	8	8	1.14E+00	9.22E-01	*
N4-c8	8	1.71E-04	N3fem	7	7	1.34E+00	1.34E+00	*
N4-c9	8	1.32E-03	N4fem	8	8	2.44E+00	2.44E+00	*
N4-c10	8	1.06E-03	N3fem	7	7	1.41E+00	1.08E+00	*
N4-c11	8	1.06E-03	N3fem	7	7	1.41E+00	1.08E+00	*
N4-c12	8	2.11E-04	N3fem	7	7	1.35E-01	1.35E-01	*
N4-c13	8	1.36E-03	F8	8	8	4.09E-01	4.09E-01	*

Table 5.9 Nest 6 Female parentage assignment within her nest.

Offspring ID	O loci typed	Prob. non-exclusion	Candidate parent ID	CP loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N6-c1	7	1.19E-02	N6fem	7	7	3.30E+00	3.30E+00	*
N6-c2	7	2.52E-03	N3fem	7	6	2.37E+00	2.08E+00	*
N6-c3	7	3.19E-03	N6fem	7	7	2.70E+00	2.70E+00	*
N6-c4	7	7.25E-04	N2f	8	7	-4.61E-01	0.00E+00	
N6-c5	7	4.38E-03	N2f	8	7	6.53E-01	6.53E-01	*
N6-c6	7	5.95E-03	N6fem	7	7	3.60E+00	3.60E+00	*
N6-c7	6	2.88E-02	N6fem	7	6	2.52E+00	2.52E+00	*
N6-c8	7	7.80E-03	N6fem	7	7	3.29E-01	3.29E-01	*
N6-c9	6	2.55E-02	N6fem	7	6	2.04E+00	9.33E-01	*
N6-c10	7	5.80E-04	F8	8	7	7.76E-02	7.76E-02	*
N6-c11	7	2.64E-04	N6fem	7	7	9.94E-01	4.93E-01	*
N6-c12	6	2.74E-02	N4fem	8	6	1.15E+00	3.26E-01	*
N6-c13	7	5.26E-03	N6fem	7	7	3.61E+00	3.42E+00	*
N6-c14	7	2.06E-02	N6fem	7	7	2.32E+00	9.43E-01	*
N6-c15	7	1.14E-02	N3fem	7	6	2.46E+00	1.02E+00	*
N6-c16	7	4.67E-04	N6fem	7	7	1.77E+00	1.77E+00	*
N6-c17	7	3.33E-03	N3fem	7	6	2.38E+00	1.40E+00	*
N6-c18	7	1.64E-03	N2f	8	7	-6.16E-01	0.00E+00	

Table 5.10 Nest 7 female parentage assignment

Offspring ID	O loci typed	Prob. non-exclusion	Candidate parent ID	CP loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
N7-c1	8	7.51E-04	N7fem	8	8	2.23E+00	2.23E+00	*
N7-c2	8	5.38E-05	N4fem	8	8	1.88E-02	1.88E-02	*
N7-c3	8	3.04E-05	N7fem	8	8	1.51E+00	1.51E+00	*
N7-c4	8	1.23E-04	N6fem	7	7	7.04E-01	2.56E-01	*
N7-c5	8	1.60E-05	N7fem	8	8	7.66E-01	7.66E-01	*
N7-c6	8	3.20E-05	N7fem	8	8	5.05E+00	5.05E+00	*
N7-c7	8	2.43E-04	N7fem	8	8	2.91E+00	2.48E+00	*
N7-c8	8	2.06E-04	N7fem	8	8	-1.49E-01	0.00E+00	
N7-c9	8	9.29E-05	N7fem	8	8	3.14E+00	3.14E+00	*
N7-c10	7	2.64E-05	N4fem	8	7	-6.68E-01	0.00E+00	
N7-c11	8	6.62E-05	N7fem	8	8	9.15E-01	9.15E-01	*
N7-c12	8	4.11E-04	N7fem	8	8	3.64E+00	3.64E+00	*

Table 5.11 Massai Ostrich Farm family female parentage assignment

Offspring ID	O loci typed	Prob. non-exclusion	Candidate parent ID	Candidate Parent loci typed	Offspring Candidate Parent loci compared	LOD	Delta	Confidence
MOF-C2	5	1.11E-03	MOF-F3	5	5	1.96E+00	1.04E+00	*
MOF-C4	5	6.55E-03	MOF-F4	5	5	1.73E+00	2.26E-01	+
MOF-C5	5	8.60E-03	MOF-F3	5	5	1.68E+00	4.31E-01	+
MOF-C6	5	6.58E-03	MOF-F2	5	5	2.23E+00	2.23E+00	*
MOF-C7	5	9.99E-03	MOF-F2	5	5	7.60E-01	1.08E-01	+
MOF-C8	5	8.69E-03	MOF-F1	5	5	2.03E+00	1.58E+00	*
MOF-C9	5	3.39E-03	MOF-F2	5	5	1.24E+00	1.24E+00	*
MOF-C10	5	5.36E-03	MOF-F1	5	5	1.91E+00	1.91E+00	*
MOF-C11	5	8.27E-03	MOF-F2	5	5	8.58E-01	6.79E-01	+

Table 5.12 Massai Ostrich Farm family male parentage assignment

Offspring ID	Offspring loci typed	Prob. non-exclusion	Candidate parent ID	Candidate Parent loci typed	Offspring-Candidate Parent loci compared	LOD	Delta	Confidence
MOF-C2	5	1.11E-03	MOF-M1	5	5	9.23E-01	4.61E-01	+
MOF-C4	5	6.55E-03	MOF-M1	5	5	6.89E-01	2.27E-01	+
MOF-C5	5	8.60E-03	MOF-M1	5	5	1.07E+00	1.07E+00	*
MOF-C6	5	6.58E-03	MOF-M1	5	5	1.30E+00	1.30E+00	*
MOF-C7	5	9.99E-03	MOF-M1	5	5	1.30E+00	4.74E-01	+
MOF-C8	5	8.69E-03	MOF-M1	5	5	1.12E+00	9.01E-01	*
MOF-C9	5	3.39E-03	MOF-M1	5	5	7.23E-01	7.23E-01	+
MOF-C10	5	5.36E-03	MOF-M1	5	5	1.84E+00	1.84E+00	*
MOF-C11	5	8.27E-03	MOF-M1	5	5	1.27E+00	5.05E-02	+

Table 5.13

Reproductive output (95% confidence) per nest for sampled candidate parents. N3, N4, N6, and N7 refer to nests 3, 4, 6 and 7 respectively.

	Nest 3	Nest 4	Nest 6	Nest 7	Pair-bond reproductive output
N3 male	9	0	4	0	2
N3 female	2	5	3	0	2
N4 male	1	8	1	0	3
N4 female	0	5	1	1	3
N6 male	0	4	8	0	4
N6 female	4	0	10	1	4
N7 male	3	0	1	10	7
N7 female	2	0	0	8	7
Unsampled males	5	0	4	2	-
Unsampled females	2	0	2	2	-

Table 5.14. Fertilisation success of different mating strategies by the territorial male and major female across the nests analysed. EPP(I), EPP(E), EPP, EPM(I), EPM(E), EPM, IPP and IBP are defined in the text.

	Nest 3	Nest 4	Nest 6	Nest 7
EPP(I)	7	5	4	3
EPP(E)	4	1	4	4
EPP	11	6	8	7
EPM(I)	0	2	6	1
EPM(E)	8	1	5	2
EPM	8	3	11	3
IPP	2	4	4	7
IBP	9	3	4	1

Table 5.15 Incubated fertilised eggs of individuals sampled at Nairobi National Park.

Individual ID	N3 female	N4 female	N6 female	N7 female	N2 female	N8 female	Unsampled females
N3 male	2	0	5	2	0	3	1
N4 male	2	3 (1*)	0	0	0	4	0
N6 male	6	1	4	0	1	0	0
N7 male	0	0	3	7	1	0	3
Unsampled males	0	2	3	1	1	3	2

* represents eggs fertilised by the pair-bond but laid in another nest.

Table 5.16 Total individual reproductive output of nesting pair-bonds

N3 male	N3 female	N4 male	N4 female	N6 male	N6 female	N7 male	N7 female
13	10	10	7	12	15	14	10

5.3.1.3 Intraspecific brood parasitism

Eggs not belonging to either of the nesting individuals were designated as intraspccific brood parasitism (IBP). A total of 17 (27.9%) eggs of the incubated clutches were intra-specific brood parasitic eggs. There was a large variation in the occurrence of IBP ranging from 1 egg (8.3%) in nest 7 to 9 eggs (50%) in nest 3 (Table 5.12).

5.3.1.4 Ostrich reproductive output variance

I was unable to collect large samples encompassing purely minor females and non-territorial males that were in breeding condition. However, the single Maasai Ostrich Farm family sample might give an insight into variance in reproductive success in the ostrich. This family, consisting of 2 males and 4 females, had been restricted to its own pen. Of the two males, both in breeding condition, one male achieved 100% reproductive success as he fathered all the nine chicks (Table 5.11). The females however all had some reproductive success with 2 (22.2%), 4 (44.4%), 2 (22.2%) and 1 (11.1%) chicks being mothered by female 1, 2, 3 and 4 respectively (Table 5.12). Further studies, recording matings and analysing sperm fertility would need to be carried out.

5.4 Discussion.

Variation in avian mating systems has the potential to advance our understanding of the balance between reproductive conflict and cooperation. Such variation raises a number of questions: are the different mating patterns within a population adaptive to individuals exhibiting them? What determines the mating pattern practised by particular individuals? Mating systems may be determined by ecological features of animals' environments (Emlen & Oring, 1977) or by conflicts of interest between individuals (Trivers 1972; Davies 1989). Here I examined how conflicts of interest are resolved in the complex and unique ostrich breeding system.

Ostrich breeding is characterised by serial polygynous mating, communal laying and monogamous parental care system. Only the major female, who initiates laying, and the territorial male incubate the eggs. Our mainly molecular genetic approach is in agreement with Hurxthal's (1979) and Bertram's (1982) field observations that 2-16 females lay 15-67 eggs in a nest. Depending on the assumption made about the parentage assignment to unsampled females (i.e the same or different individuals), this study estimated that 3-7 (figures 5.7-5.10) females lay 18-66 (table 3.1) eggs in the same nest.

Predictions

1. Major females are able to ensure that their eggs are incubated.

With the pair constrained to incubate only 20-21 of these eggs (Hurxthal 1979, Bertram 1982) the major female is thought to favour retention of her own eggs within the central clutch for incubation. Excess eggs are ejected, by the major female, to the peripheral clutch 1-2 metres away where they remain unincubated and perish (Bertram, 1979). Bertram suggested that the birds were using cues such as shape, size or pore pattern to distinguish between eggs. Out of 5 nests examined, he identified the major females' eggs by morphological similarity and concluded that only 1 putative major female's egg was ejected onto a peripheral clutch. Our molecular findings indicate that the females may be able to select their eggs for retention in the central clutch for incubation. However, this may be due to disproportionate laying of eggs by the major and minor females within a nest rather than egg ejection by the major female. Data from nest 3 where the apparent major female lay only 2 eggs further confounded the results. Exclusion of this female from analysis

reveals that the major female does have a higher number of eggs retained in the central clutch for incubation ($\chi^2_{(3)} = 9.39$ P=0.02). However, data from peripheral eggs would be necessary for unambiguous verification. The selection process continues throughout the laying process with eggs being moved between the peripheral and central clutches interchangeably (Bertram 1992).

2. Most of the major females' eggs are fertilised by the resident territorial males.

Of the major females' eggs laid in their clutch a mean of 4.25 ± 2.06(SD) were fertilised by the resident territorial males (IPP) while 2.25 ± 2.63(SD) were fertilised by other territorial males (EPM(I) (Table 5.12). Though the resident territorial males achieved nearly twice the level of fertilisations, this was not statistically significant (t test, t=1.22, df=3, P = 0.31). It does however indicate that there was a fertilisation success skew in favour of the resident male who was as successful as the sum of all the other males that mated with the major female.

3. The resident territorial males fertilise some, but not all, of the other females' eggs in the nest.

The results show that the male did fertilise some, but not all of the eggs of the minor females that were incubated in his nest. In their nests, the resident male managed to get the same amount of minor female eggs fertilised (EPP(I), 4.75±1.71 (SD) as did the other males combined (IBP), 4.25±3.40 (SD). The preferential mating with new females entering his territory is a strategy that achieves a reproductive payoff from the minor females that deposit their eggs in his nest. However, not all males achieved that same level of extra-pair fertilisation success within their nests. Male nest 3 fertilised seven of the minor females' eggs laid in his nest while male nest 7 fertilised three.

Sexual selection exhibited as intra-sexual male-male competition occurs in the ostrich despite the presence of a monogamous parental care system. Territoriality in the ostrich is evidence of male competition for access to females.

Major females lay as minor females before starting their own nest, after losing their nest or, on very rare occasions, concurrently as a major female (Bertram 1992). Molecular evidence carried out here on major females that also lay in other nests as minor females suggests that they commonly lay as minor females before starting a

nest and becoming a major female. With the exception of one egg laid by female 4, none of the eggs of major females fertilised by their male were deposited outside the pair bond nest.

This study reveals a complex ostrich mating pattern that is primarily polygynandrous, both sexes mating with multiple partners. It lends support to Hurxthal's (1979) observational studies suggesting serial polygyny. Hurxthal (1979) observed that females, who have a large home range relative to the males' territories, move through 4-7 male territories and may mate with the territorial male in his territory. Ostriches copulate repeatedly and resident territorial males copulate preferentially with new females entering their territory (Hurxthal, 1979). This is probably a strategy of increasing the reproductive payoff through extra-pair fertilisations while reducing the likelihood of being cuckolded. I have found that the resident male fertilised as many major and minor females' eggs incubated by him as all the other territorial males combined. These minor females laid in his nest, benefiting from his parental care or in other males' nests, in which case he was parasitising the parental investment of other males.

Division of the clutch into incubated and peripheral clutch is carried out by the female to her benefit as discussed earlier. That the males do not eject any eggs is not surprising. The "quasi parasitism"

hypothesis supposing that the host males are fathers to the parasitic chicks (Wrege & Emlen 1987) is partly supported here by the fast that the resident territorial males fertilised approximately half the eggs laid by minor females in his nest. Their fertilisation of eggs from a large number of females make the males unlikely to identify eggs fertilised by them and also make it likely that their genetic contribution is large. Indeed, males have never been observed to eject eggs from the central clutch (Hurxthal 1979, Bertram 1979, personal observations). In birds, the best explanation for repeated intra-pair copulation (and also in the case of the ostrich extra-pair), if initiated by the male, is that sperm so released devalues that of competing males (Birkhead & Moller 1992, Birkhead 1995a). This is common in such species as the ostrich, where opportunities for extra-pair copulation are high and is consistent with known mechanisms in birds (Birkhead *et al* 1995, Colegrave *et al* 1995)

The females have two strategies: One as a minor female, where she moves through different males' territories, mating with these males and laying eggs in their nests. The minor females' mating strategy is likely to be an insurance for the males' parental investment as a result of increases paternity assurance. It benefits the territorial male and the minor female if the eggs are incubated. It does not obviously benefit the major female. The major female would,

however, be expected to be under selection not to provide parental care if she is not related to the minor female unless she benefits in some indirect way. By laying in several nests, the minor females increase the probability that some of their eggs would be retained in the central clutch. All the females in this study got their eggs incubated in the central clutch of at least 2 of the 4 nests studied (Table 5.11).

Females also have the alternative strategy of becoming a major female by pairing up with a territorial male and contributing to parental care in their nest. The females may then able to select their eggs for retention in the central clutch for incubation (Bertram, 1979). There is some evidence of this in our molecular approach study but further investigations especially with reference to the peripheral eggs is necessary.

All the females in our study laid eggs both as major females in their own nests and as minor females away from their nests. Females may then able to maximise their reproductive success by adopting these alternative strategies. Such plasticity in adaptation may be a response to selective pressures brought about by unpredictable sub-saharan savanna bushland climate, high levels of predation or destruction of nests by large ungulates. The ability to

rapidly switch from one strategy to another or to combine them to counter possible losses in reproductive output would be invaluable.

The ostrich mating system has been termed labile (Handford & Mares, 1985) variously described as monogamous (Roberts 1958, Seigfried & Frost 1974, Sauer & Sauer, 1959) simultaneous polygynous, (Sauer & Sauer 1966) sequentially polyandrous or polygynandrous (Hurxthal 1979, Bertram 1982). Indeed Hurxthal (1979) termed it facultative monogamy since the monogamous aspects appeared most basic to the system. The occurrence of variable mating patterns, including mate sharing by both males and females may have no single explanation. In birds, variable mating systems have been recorded in The Native Tasmanian hen *Gallinula mortierii* (Maynard Smith & Ridpath 1972, Goldizen *et al* 2000), Acorn woodpeckers *Melanerpes formicivoros* (Koenig & Muume 1987), Galapagos Hawk *Buteo galapagoensis* (Faaborg *et al* 1995), Dunnock *Prunella modularis* (Davies 1985, 1992, Birkhead et at 1991), Alpine Accentor *Prunella collaris* (Nakamura, 1990) Smith's Longspur *Calcarius pictus* (Briskie 1992, 1993) Superb Fairy-wren *Malurus cyaneus* (Mulder & Cockburn 1993), Aquatic Warbler *Acrocephalus paludicola* (Schulze-Hagen *et al.* 1995) and the Hihi *Notiomystis cincta* (Castro *et al* 1996).

Variable mating systems may or may not present a conflict between the sexes. This depends on the male and female optimal mating patterns. For example, a lack of conflict is evident in the native Tasmanian hen where related males share mates and offer females a quality territory (because more males can defend proportionally larger territories) thereby both sexes increase their reproductive success (Maynard Smith & Ridpath 1972 Goldizen *et al* 2000). A conflict may arise where the sexes have different optimal mating patterns. In the Dunnock *Prunella modularis* and alpine accentor *Prunella collaris* variable mating patterns reflect conflicts of interest between the sexes with dominant males seeking to monopolise copulations while females may seek copulations from several males to gain their parental investments (Burke 1989, Briskie 1992, Davies 1992, Hartley *et al.* 1995).

In the ostrich, *S. c. maasaicus*, conflicts of interest arise between the male and female resulting in different strategies aimed at maximising individual reproductive success. Males attempt to maximise their reproductive success by establishing a nesting territory, a monogamous parental pair bond with a major female while copulating with new females entering his territory who lay as minor females in his nest. Females also attempt to increase their reproductive success by occasionally alternating their strategy from

minor to major and vice versa depending on ecological conditions (e.g. predation and mate availability). She is however, more likely to start a new nest with her previous mate as a major female (Bertram 1982).

The territorial male and the major female therefore, though co-operating in parental care, seem to resolve their inter-sexual conflict by adopting different strategies aimed at maximising their individual reproductive success. We found that both males and major females achieved the same level of reproductive success: 12.25 ± 1.17(SD) for males and 10.50 ± 3.32(SD) for major females. Females are indeterminate layers if eggs are taken away on laying (Duerden 1912). This indicates that as minor females, the females may not be constrained by the number of eggs they lay. However, as major hens, constraints such as predation or reduced hatchability (Gonzalez 1999) may constrain the number of eggs a major female lays prior to incubation. The alternative strategies a female can employ, coupled with a monogamous parental care system, may then raise the reproductive success close to the male's.

Thus there is an indication of an adaptive resolution to the inter- and intrasexual conflict of interest, arising from the unique ostrich breeding system that is characterised by polygynandrous mating, communal nesting but monogamous parental care with

individuals being selected to maximise their own individual reproductive pay off, even at a cost to their mate.

Major females may favour their own eggs for incubation. They also retain other minor females' eggs for incubation and allow unhindered access to their nests for laying. Even if the major female was constrained to lay 10 eggs, incubation of the extra eggs would carry energetic or other costs. Bertram (1992) suggested several explanations for acceptance of minor females: physical conflict would have a high cost for the major female, given her high initial investments e.g. damage of eggs in the clutch, predation risks as a consequence of damaged nest due to predator attraction by smell or attraction of predator by the conflict. It may also be that there are reproductive benefits such as decreased predation due to prey dilution or inclusive fitness benefits secondary to helping relatives. The next chapter investigates kin selection as a possible explanation of the co-operation between the major and minor females in ostrich communal nesting.

Further work should be carried out extending the reproductive analyses to females that undertake a purely minor female role and assessing the contributions of individuals in the peripheral clutch. If studied over several seasons and across more nests, a more comprehensive overview of the total costs and benefits of the

individuals interacting in the communal nesting system of the ostrich will be gained.

Chapter 6

THE OSTRICH SEX RATIO

6.1 Introduction

The observed sex ratio in a population is as a consequence of the primary sex ratio or secondary sex ratio adjustments. The primary sex ratio is the sex ratio with which each generation begins. Ideally it is the sex ratio among fertilised eggs (Trivers, 1985). Secondary adjustment results from secondary factors adjusting the primary sex ratio such as differential mortality e.g. arising from increased male-male competition or, in sexually dimorphic species, differential predation due to male elaborate secondary sexual characteristics.

The breeding system exerts an influence on the secondary sex ratio in a population. In animals, most studies show an unbalanced sex ratio within the population. This unbalanced sex ratio, with the exception of monogamous birds, is usually biased in favour of females (Trivers 1985). This is attributed to males suffering higher mortality than females (Trivers 1972) as evident in insects (Cornert et al 1960, Rockstein 1959), fish (Beverton 1959), reptiles (Tinkle 1967) and many mammals (Myers 1971, Wood 1970). In monogamous birds the sex ratio bias is in favour of males, unlike

polygynous or promiscuous species where there are fewer males (Mayr 1939). Promiscuous or polygynous species with little parental investment have higher male differential mortality than monogamous males with parental care (Lack 1968). Differences in relative parental investment result in differential mortality depending on the reproductive strategy an animal takes with the most adaptive being the strategy with the maximum lifetime reproductive success.

In animals with little or no male parental investment, the males suffer higher mortality as they expend resources as reproductive effort in intrasexual competition. This is evident in polygynous mating systems where the male offers little more than his sperm e.g. red deer *Cervus elaphus* (Rose *et al* 1998), elephant seals *Mirounga angustirostris* (Clinton 1993) and greater kudus *Tragelaphus strepsiceros* (Owensmith 1993). Where males offer parental investment of over one-half but less than that offered by the female, as is common in birds, male mortality is lower than the female's (Payevsky 1997). This may be due to a disproportionate lowering of the reproductive effort expended in male-male competition in relation to increased parental investment (Trivers 1972).

Adjustment of the primary sex ratio (i.e. sex allocation at birth (in viviparous taxa) or oviposition (in oviparous taxa)) at the family level, if under selection and therefore adaptive, would be expected to

influence the population sex ratio (Frank 1990). Across diverse animal taxa females may adjust the offspring sex ratio in favour of the sex that will increase their future fitness. Fisher's (1930) sex ratio theory predicted that there would be evolutionary stability of the sex ratio if the parent controlling the sex ratio derived the same fitness from offspring of both sexes and neither sex required greater effort to produce. If one sex was rarer, then the mating success of this rare sex would be greater, offering fitness advantages. That sex would be preferentially produced until the advantage is lost at equilibrium whence the selective pressure for overproducing either sex ceases.

Subsequent modifications of Fisher's original theory show that if the equal returns condition is violated and fitness benefits therefore vary e.g. due to differential relatedness (Trivers & Willard 1973, Trivers & Hare 1976), energetic demands, reproductive value (Dijkstra *et al* 1990) and non-random mating (e.g. Pamilo 1990, Bouke & Frank 1995, Crozier & Pamilo 1996), then the stable numerical sex ratio may be expected to differ from equality.

Testing sex ratio patterns in vertebrates, particularly in birds has been difficult because obtaining sex ratio data before significant mortality has occurred is difficult. Further, the theory predicting sex allocation patterns is complex (Sheldon 1998) given the constraint of chromosomal sex determination and the presumably low ability of

the parent influencing sex ratio adjustment to predict factors that determine this adjustment. Such factors may be availability of resources such as food in the future or additive genetic components of ornaments (determining quality or attractiveness of mate).

Even where the 1:1 sex ratio is an evolutionary stable strategy, subtle selection favours any parents who can adjust the sex ratio to produce the sex with a higher reproductive success (Trivers & Willard 1973, Trivers 1985, Clutton-Brock 1984). Some recent empirical evidence in birds suggest differential sex ratio allocation in relation to the external environment. Tawny owls in food abundant areas (high vole density) had broods overproducing larger females whose future reproductive success was influenced by the availability of food as nestlings. Males are smaller and their reproductive success was not affected to the same degree (Appleby *et al.* 1997). In diurnal raptors, Daan *et al.* (1996) showed that the sex allocation pattern was correlated with the date of hatching, depending on which sex bred in the succeeding year as opposed to waiting an additional year. The sexes of chicks that hatched earlier in the season were in favour of the sex that bred sooner.

The social environment may also affect sex allocation. In co-operative breeding birds, one sex, usually male, may remain in the natal territory and help rear siblings (Sheldon 1988). Subtle sex

allocation may occur depending on food resources (i.e ability of territory to support additional adults) or current presence of helpers on the nest. Using DNA from nestlings Komdeur *et al.* (1997) showed such a result within family biases in the primary sex ratio of the Seychelles warbler *Acrocephalus sechellensis*. If the habitat is saturated with breeding pairs, helpers (usually daughters) have limited dispersal opportunities therefore helping is frequent. Daughters of parents in poor habitat are costly presumably because they deplete insect prey. Parents breeding in poor quality territories produced 77% sons as opposed to those in high quality territories which produced only 13% sons (Komdeur *et al.* 1997). This biasing was as expected. Experimental manipulations to change territory quality resulted in an expected change in the sex ratio.

The sex ratio may vary in relation to parental quality. If parental quality results in higher reproductive success, it would be predicted that the offspring would be biased in favour of that sex. The reproductive success of male collared flycatchers *Ficedula albicollis* is positively correlated with the white forehead patch. The trait is heritable and under directional selection due to female preference (Sheldon et al 1997). Broods from males with large forehead patches were, as expected male-biased (Ellegren *et al* 1996). In polygynous mammals, the prediction would be that maternal investment should

depend on the relative resource status of the female in relation to sex-specific demands of the offspring i.e. females should bias sex allocation depending on their body condition (Kohlmann 1999). In red deer, *Cervus elaphus* females in better body condition produced significantly more sons, who are energetically more demanding but would be expected to have higher reproductive success (Clutton-Brock *et al.* 1984, Kohlmann 1999).

The mechanisms that control sex allocation at oviposition, the primary sex ratio adjustment, in female birds is unknown. The mechanisms employed may be control of meiotic segregation (Dijkstra *et al.* 1990, Ellegren *et al.* 1996, Lessells *et al.* 1996, Anderson *et al.* 1997, Kilner 1998), selective resorption or dump laying of the wrong sex ova (Emlen 1997), or waiting until the right sex ova is released before starting a clutch (Emlen 1997). The latter situation would mean that only birds with small clutches would effectively control sex bias (Such as Komdeur's Seychelles warblers with a mean clutch size of 1).

In the ostrich, a slight female-biased population sex ratio of 1:1.1 (Hurxthal 1979) and 1:1.4 (Bertram 1982) has been reported. Here I set out to determine if this is also found in the primary sex ratio. Any primary sex ratio deviation from equality may be a consequence of: 1) Adjusting the conception sex ratio in favour of the

less common males. 2) Producing the less expensive sex. 3) Producing, at fertilisation, the sex that experiences increased mortality during parental investment. At the end of the parental investment period, the sex ratio should be biased in the opposite direction (Trivers 1985). Since we have no evidence in the ostrich for the latter 2 or 3, here I set out to investigate the primary sex ratio with the expectation that females would produce more of the less common males.

With the exception of the slight female bias in the population sex ratio, I had no data on any aspects of parental quality, social or external environment that may influence the primary sex ratio.

The genetic sex in many species of birds cannot be determined by morphology at hatching. Sex identification at the DNA level is now widely used. It is accurate, fast, inexpensive and can be carried out at any stage of the life history. The isolation of the avian CHD gene (Ellegren 1996, Griffiths *et al.,* 1996) has facilitated ease in sex characterisation in non-ratite birds. In ratites this has proved difficult since incomplete differentiation of the sex chromosomes has meant a paucity of unique sequences or markers located in the W-chromosome. This problem was solved by the identification of a W-linked sex-specific DNA marker in the ostrich *Struthio camelus* (Bello & Sanchez 1999). I used this marker to characterise the sex of

embryos from different nests and thus determine a primary sex ratio. I tested the prediction that the primary sex ratio is skewed in favour of males.

6.2 Methods

Using OSM 4 (Kimwele *et al.* 1998) as an internal marker, a multiplex PCR reaction was done using 50ng DNA, 2.5 µl PCR buffer, 1.5 mM MgCl2, 200 µM of each dNTP, 12.5 pmol of each primer of OSM4 and primers SS1 and SS2 (Bello & Sanchez 1999) and 0.75 U of Taq polymerase in a volume of 25 µl. The thermal cycling conditions were 95°C for 2 min, 27 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min with a final extension of 72°C for 2 min. The PCR products were then size separated by electrophoresis on a 1.5 % agarose gel containing ethidium bromide and visualised under UV light. The heterogametic female (ZW) showed 2 band, one W-linked locus amplification and the other the internal marker band. Being homogametic, only the internal marker would be amplified in males, which therefore showed a single band. Instances where non-amplification of the internal marker occurred. Such cases were re-amplified and a positive result was achieved (Figure 6.1).

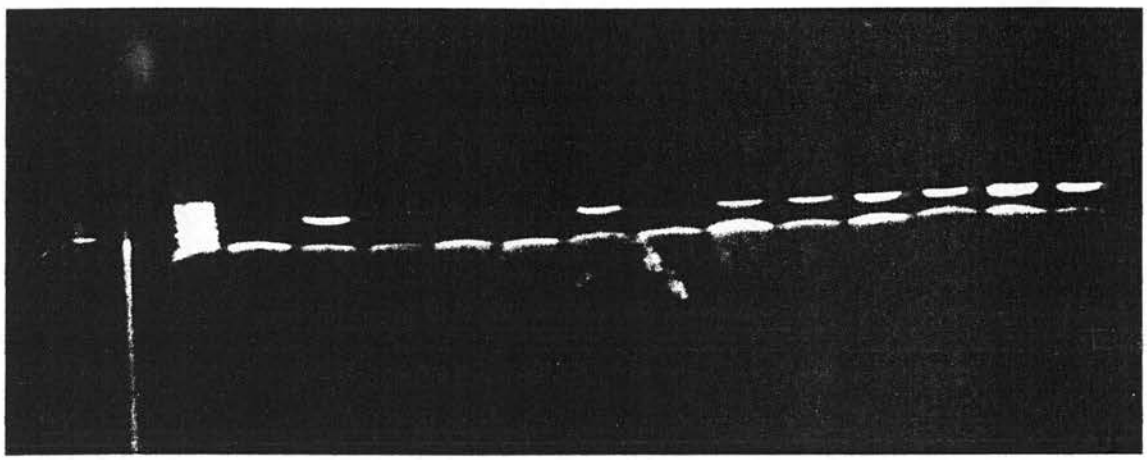


Figure 6.1: Embryonic sex determination of 13 egg membranes from nest 3, Nairobi National Park. Heterogametic females exhibit two bands, the longer one (648 bp) amplifying a W-linked locus (Bello & Sanchez 1999) and the shorter one the OSM4 internal microsatellite locus marker (approximately 130 bp) locus with the homogametic male exhibiting one band (the internal marker). Eight females and five males are identified in this figure.

6.3 Results

Sex analysis revealed 26 incubated eggs that were male and 35 that were female (Table 6.1). This represented a primary male:female sex ratio of 1:1.35, a slight skew in favour of females. This was however not statistically different form a 1:1 ratio ($\chi^2_{(1)}$=1.33 p=0.25). Because of the small sample size, the power of detecting any significant change is low.

Table 6.1 Sex ratio of incubated eggs across the nests analysed at Nairobi National Park.

Nest	Offspring sex	
	Male	Female
3	7	11
4	5	8
6	8	10
7	6	6

6.4 Discusssion

The primary sex ratio was 1:1.35. Although slightly skewed, this was not different from equality. A caveat is that there was some loss to predation once the eggs were abandoned and before we were granted permission to collect the eggs for tissue sampling (Chapter 2). I assumed that predation was random.

There was the problem of sample size. It is not clear how large a sample size is required to show significant deviations from a 50/50 (1/1) ratio. For example, a sample of 400 animals showing a 44/56 sex ratio does not deviate significantly from a 50/50 ratio, nor does it differ from a 38/62 ratio (Trivers 1978). I estimated that for the ratio of 1:1.35 detected here, I would need a sample size of 170 to detect a significant deviation from equality ($\chi^2_{(1)}$ P=0.05). A much larger sample is therefore needed to have enough power to detect a skew that is not extremely large.

A secondary sex ratio adjustment is likely. This may be explained by differential mortality due to polygyandrous mating system which predisposes the male to increased male-male competition. However, the opposing selective pressure brought about by increased male parental investment which would reduce the reproductive effort expended in male-male competition and thus

reduce the relative mortality of the male. In the ostrich, male-male competition for females has resulted in territorial defence and sexual dimorphism in plumage. Males have more conspicuous black and white coloration probably making them more prone to predation. However, the likelihood of predation is minimised by the less conspicuous grey-brown female incubating during most of the daylight hours.

Sexual bimaturation may also explain the apparent sex ratio skew. Farm data indicate that females mature at 3 yrs while males mature at 4 yrs of age (Douglass 1881). The females then join the effective breeding population earlier than the males. Consequently a female bias in the operative but not the population sex ratio may arise. However, there is as yet no empirical evidence on factors that may lead to a secondary population sex ratio skew.

In conclusion, more data should be collected to increase the power of detecting any variance of the primary sex ratio from equality within the ostrich. Further, for any such variance to be explained in terms of adaptation, ecological factors explaining the probable selective forces need to be examined.

Chapter 7

ALTRUISM IN OSTRICH COMMUNAL LAYING - KIN SELECTION HYPOTHESIS EXAMINED.

7.1 Introduction

Though evolutionary adaptation is a genetic process, it can be understood by approximating phenotypic traits. Such traits, with the highest fitness tend to be favoured (Grafen, 1982). However, some social behaviours, though phenotypically lowering fitness are selected. Such behaviours, termed as altruism, are defined as behaviours acting to increase other individuals' lifetime reproductive success at a cost to one's own survival and reproduction. The ostrich communal nesting system reflects such apparent altruism. Though the major female lays and incubates on average 9 eggs in the central clutch, up to 11 others (Hurxthal 1979, Bertram 1982) are laid by other minor females but the costs of parental care such as egg guarding, incubation, brooding and chick escorting, are borne exclusively by the major female and the territorial male. The territorial male has the potential to fertilise all these other females eggs and indeed he mates preferentially with new females venturing into his territory (Hurxthal 1979) possibly to increase his paternity

assurance and therefore his reproductive success. Here, I have shown that within his clutch, he achieves more fertilisations of these other females' eggs than do other males. The major female, who selects eggs retained in the central clutch for incubation, leaves extra eggs that are not her own. This study suggests that a major female may retain her eggs in the central clutch but still retains other females eggs for incubation. This is in agreement with Bertram's observations (1979). Apart from 6 non-assigned eggs, my analysis show that eggs in all 4 nests examined were laid by the same small group of 3-5 females (Figure 5.2). The explanation could be that:

1. There is a small number of females laying in this part of Nairobi National Park: "the limited females hypothesis".
2. The eggs of particular females are favoured "the favoured females hypothesis".
3. The major female benefits from letting any random dumped eggs to occupy the extra space due to presumed benefits such as anti-predation dilution effect. The females dumping presumably cheap eggs will benefit even if very few of them are retained in the central clutch and may not lose much if that does not happen.
4. That the female is unable to stop other females laying due to the high physical costs of a conflict such as injury,

damage to eggs and predation or due to the physical disadvantage of a sitting bird "the harrassment hypothesis".

The limited females hypothesis is unlikely since this area covered at least 70% of the park. Given that the previous KWS census put the adult population at 188, and assuming a sex ratio of 1:1 and that 20% were not sexually active (i.e. offspring from two previous years) then at least 53 sexually active females were in this area. Further, I identified a total of 8 nests and 3 others that had hatched. To test this hypothesis requires additionally, the collection of eggs from all the peripheral clutches for artificial incubation and analysis. In this study, I was unable to do this but it is possible to do so in the future.

The "mutualism hypothesis" is difficult to test as the benefits to both parties need to be quantified. Quantifying anti-predatory benefits is undoubtedly not easy. The harrassment hypothesis needs more data than the occasional harrassment that has been recorded by Bertram (1992), who observed some persistent pecking of sitting birds to the point where they got up and let the incoming female deposit her egg. Further, it seems unlikely as in most cases the major female allows other female access to the nest without any prior conflict (Hurxthal 1979, personal communication). Both mutualism

and harassment hypothesis cannot explain why only a small number of females laid in the nests sampled.

The favoured females hypothesis is a possibility. This may be as a result of females:

a) Favouring relatives and therefore accruing inclusive fitness benefits.

b) Favouring recipient females that will reciprocate the act in the future. Reciprocity cannot be ruled out as ostrich chicks associate as large coalition of creches from about 5 weeks old. The chicks associate with group members until they are sexually mature at 2 years of age (Hurxthal, 1979). This association may allow for individual recognition and the possibility of repeated future interactions. Lifetime reproductive interactions studies need to be carried out to test the reciprocity hypothesis.

With the current available data, I am able to test a) but not b).

7.2 Kin selection

Maynard Smith (1964) coined the term kin selection to describe the process favouring characteristics that increase the fitness of close relatives, both offspring and non-descendant kin.

Parental care of own offspring is the most obvious example of altruism. Since individuals are selected to maximise their gene contributions to future generations, care of offspring, though phenotypically altruistic, is genotypically selfish as the offspring carry copies of their parent's genes. Just as gene proliferation can occur through parental care, so can it through the care of siblings and other close relatives. Helping a sibling results in the same number of gene copies being passed on as would helping an offspring. Fitness then, can be gained either through individual reproduction as direct fitness or through aiding non-descendant kin as indirect fitness (Brown, 1980). If the benefit from both direct and indirect pathways to fitness (Brown, 1987) is sufficiently large, the benefits might outweigh any costs associated with performing a seemingly risky behaviour and the behaviour will evolve and/or be maintained (Blumstein & Armitage, (1998)

Hamilton (1964) termed the benefits from both direct and indirect fitness as an individual's inclusive fitness. He described inclusive fitness as personal fitness, which an individual expresses in its production of an adult offspring, after the individual is stripped of all components considered to be due to the individual social environment and then augmented by certain fractions of the quantities of harm and benefit which the individual himself causes to

the fitness of his neighbours. Hamilton realised the importance of these fractions to the evolution of altruism. These fractions are represented by the coefficient of relatedness (r), and quantifies relatedness by measuring the probability that the affected individuals will share copies of genes above and beyond random levels. According to Hamilton's rule, a behaviour is favoured by selection when;

$$\Delta w_x + \Sigma r_{yx} \Delta w_y > 0 \qquad (1)$$

Where,

Δw_x

Is the change the behaviour causes in the actors fitness.

Δw_y

Is the change the behaviour causes in it's partner's fitness.

and

r_{yx}

Is their genetic relatedness.

The left hand side is the inclusive fitness effect of the behaviour; it tallies up all the fitness effect of the act after first devaluing each by the probability that the affected individual shares genes for the

behaviour above and beyond random levels (Queller & Goodnight, 1989).

This may be simplified to,

$$rB - C > 0 \qquad (2)$$

Where, r = relatedness coefficient

C = cost to altruist

B = benefit to recipient

It may be beneficial to measure benefits as offspring gained or lost. In this case, the behaviour is favoured if;

$$\frac{B}{C} > \frac{r \text{ altruist to own offspring}}{r \text{ altruist to recipient offspring}} \qquad (3)$$

There are numerous examples of kin selection acting in nature are found in diverse animal orders ranging from social insects to birds and mammals . Worker bees show extreme altruism, helping rear their siblings even though they themselves are sterile, they aggressively defend the swarm and sting using their barbed stings which dislodge, leading to a certain death. In ground squirrels, *Spermophilus beldingi*, females stay in the natal territory while males

disperse. The females help their mothers and sisters in parental care as well as defence of their burrow and use alarm calling as a warning against the presence of predators (Sherman 1981). Similarly, philopatric female black tailed prairie dogs, *Cynomys ludovicianus*, co-operate with relatives to defend their territory. In the lion, *Panthera leo*, coalitions show reproductive success skew in favour of the dominant male, with the subordinate males achieving little or no success; the individuals involved are usually brothers (Packer *et al*, 1991). Female baboons (*Papio cynocephalus ursinus*) groomed maternal kin at significantly higher rates and for significantly longer periods than they groomed other females (Silk *et al*, 1999). A few examples in birds include the Tasmanian native hen, *Tribonyx mortierii*, where brothers co-operate in breeding with a single female and the trio all provision for the brood (Maynard Smith & Ridpath 1972). In Seychelles warblers *Acrocephalus sechellensis*, helpers are more likely to help full siblings than half-siblings (Komdeur 1994). Helpers in the co-operatively breeding bell miner (Conrad 1998), western bluebird (Dickinson 1996) and Arabian babblers (Wright 1998) are close relatives to the recipients of their help. In all more than 90% of 107 family living avian species so far investigated exhibit cooperative breeding where an adult member of a social

group provides regular care to offspring that are not genetically its own (Emlen, 1995).

Here, we investigate kin selection as a likely process driving altruism in the ostrich communal nesting system. Since the chicks associate in social groupings, there is a likelihood of kin recognition, though high mortality rates of up to 90% in the first year reduce this possibility. Relatives may co-operate to enhance each other's reproductive output, increasing their inclusive fitness. In measuring inclusive fitness, we should ideally measure all the various fitness consequences of the interactants, stripping off the effects of other individuals to the altruist fitness and then devaluing the recipient's fitness by *r* (Hamilton 1964; Creel 1990). Stripping is unnecessary if the interactions are conditional (where the recipient choices depend on the donor) and therefore the effects are non-additive (Queller 1996). Stripping would require that we sample the total eggs in a clutch (both peripheral and central) and therefore be able to measure the fitness consequences of all interacting individuals. Unfortunately, our attempt to do so failed as incubators were unavailable. Since the costs and benefits cannot be determined, we here assume that the benefits and costs for the interactants are the same and therefore use *r* to estimate the genes common to the interactants. Interactants with a

high r value will be maximising their indirect fitness as the recipient's fitness is devalued to a lesser extent.

Here I set out to test the kin selection hypothesis using genetic information from 8 hypervariable microsatellite marker loci. I used a relatedness index as applied by Queller & Goodnight (1989). De Ruiter and Geffen (1998) inferred relatedness, on the basis of 11 blood protein markers, using the Queller and Goodnight index of relatedness in a macaque (*Macaca fasciculatus*) population where the estimate so obtained reflected independently determined pedigree relationships. Here, I used *r* as an indirect indicator of inclusive fitness benefits.

7.3 Predictions

1. That the minor females, whose eggs are retained in the central clutch for incubation, are closely related to the major female.
2. That the major female is a close relative to those chicks, other than her own offspring, that she incubates in her nest.

7.4 Methods

Field studies, sample collection and laboratory analysis were carried out as discussed in Chapter 3. Eight loci were typed in a total of 86 individuals, 25 adults and 61 putative offspring from 4 nests.

The coefficient of relatedness r was estimated using Queller and Goodnight (1989) Relate 5.0 software program. The basic from of the relatedness regression calculation is:

$$R = \frac{\sum_x \sum_k \sum_l (P_y - P^*)}{\sum_x \sum_k \sum_l (P_x - P^*)}$$

Where

x is the index for the individuals in the data set

k is the index for loci

l is the index for allelic position

The variables in the ratio are:

P_x : The frequency within the current x individual of the allele found at x's locus k and allelic position l. This is a probability value and in a diploid must be either 0.5 or 1.0.

P_y : The frequency of that same allele in the set of partners of x – the individual(s) to which you want to measure x's relatedness. It may take the value 0, 0.5 or 1

P^* : The frequency of the allele in the population at large, with all putative relatives of x excluded as a bias correction.

R is interpreted as the number of genes (represented by microsatellite loci) that are identical by descent. Data input and specifications for calculations were followed as per their manual.

Bias correction

The population allele frequency was bias corrected by exclusion of the current *x* individual and all its close putative relatives. Therefore, I excluded all the offspring from the population allele frequency estimation. In cases where relatedness to a particular major females was being considered, bias correction was carried out by excluding offspring of the major female in question. This correction was necessary because, in a limited sample size the genetically similar relatives will bias the population allele frequencies in *x*'s direction resulting in an underestimate of relatedness. As the data set increases (i.e. number of separate sets of relatives), the effect of this bias decreases. Bias correction therefore eliminates a downward bias for small sample sizes.

Jackknife standard errors

Since the relatedness coefficient values are usually not normally distributed, the jackknife resampling procedure was used to estimate standard errors and confidence intervals. The jackknife

method involves dropping independent data points in turn, and calculating a new statistic with each reduced data set (Sokal & Rohlf 1981). These results are then processed into pseudovalues from which a standard error can be calculated. This standard error has been shown to correctly approximate the standard deviation of the means (Pamilo 1984). Jackknifing over groups is preferable (Pamilo 1984). However, I opted to jackknife over loci because where group numbers are small, as was the case in our study, the jackknife estimate over groups becomes less reliable (usually overly conservative) (Queller & Goodnight 1989).

Relatedness estimation

86 individuals were sampled. These comprised 61 chicks from 4 nests and 25 adults, including the 8 nesting individuals.

Relatedness was estimated:

1. Among the females that laid in a given nest.
2. Between the major female of a given nest and the recipient chicks within her nest.
3. Among chicks in a given nest.

We estimated relatedness in two ways:

1. **Average relatedness**

The average relatedness of small subsets of interest within the population was estimated. Standard errors and confidence intervals were found by jackknifing over 8 marker loci.

2. **Pairwise relatedness estimate**

This estimates the relatedness of one individual to another. Pairwise relatedness was carried out between individuals of the entire data set. Individual estimation of relatedness was shown by Queller & Goodnight (1989) to be highly variable in simulations using 1,500 half- and full-sibs respectively and 3 loci, each with 3 equally common alleles. This variation is due to inherent sampling error arising from the fact that related individuals are descendants of a few parents, whose genetic variability may be limited. This variability in relatedness estimate is expected to be limited if a great deal of genetic information is available. The use of eight hypervariable loci in our study is expected to provide sufficient genetic information. However, these estimates can, in aggregate, still be very useful as data in nonparametric tests.

The pairwise relatedness estimates have the following advantages

1. A downward bias for small samples is eliminated by introducing bias correction, involving exclusion of individuals closely related to the *x* individual.
2. It improves estimation of relatedness for subsets within the population. This is achieved by estimating P* separately for each group.
3. It allows estimation of relatedness for a single group or for a single pair of individuals. Though variable, these measures are useful data to apply to non-parametric tests.

7.5 Results

7.5.1 Pairwise relatedness

Co-operating females' relatedness

Figure 1 shows the bias corrected mean symmetrical pairwise relatedness among co-operating females in the 4 nests analysed. The median population relatedness was 0.01. The population pairwise relatedness ranged from -0.38 to 0.56 and the interquartile range was -0.09 to 0.13. The mean pairwise relatedness was -0.15, -0.10, -0.10 and -0.05 between females whose eggs were incubated in nests 7, 3, 6 and 4 respectively (Figure 7.1). The numbered arrows represent relatedness among interactants of a given nest.

Figure 7.1: Co-operating females mean pairwise relatedness

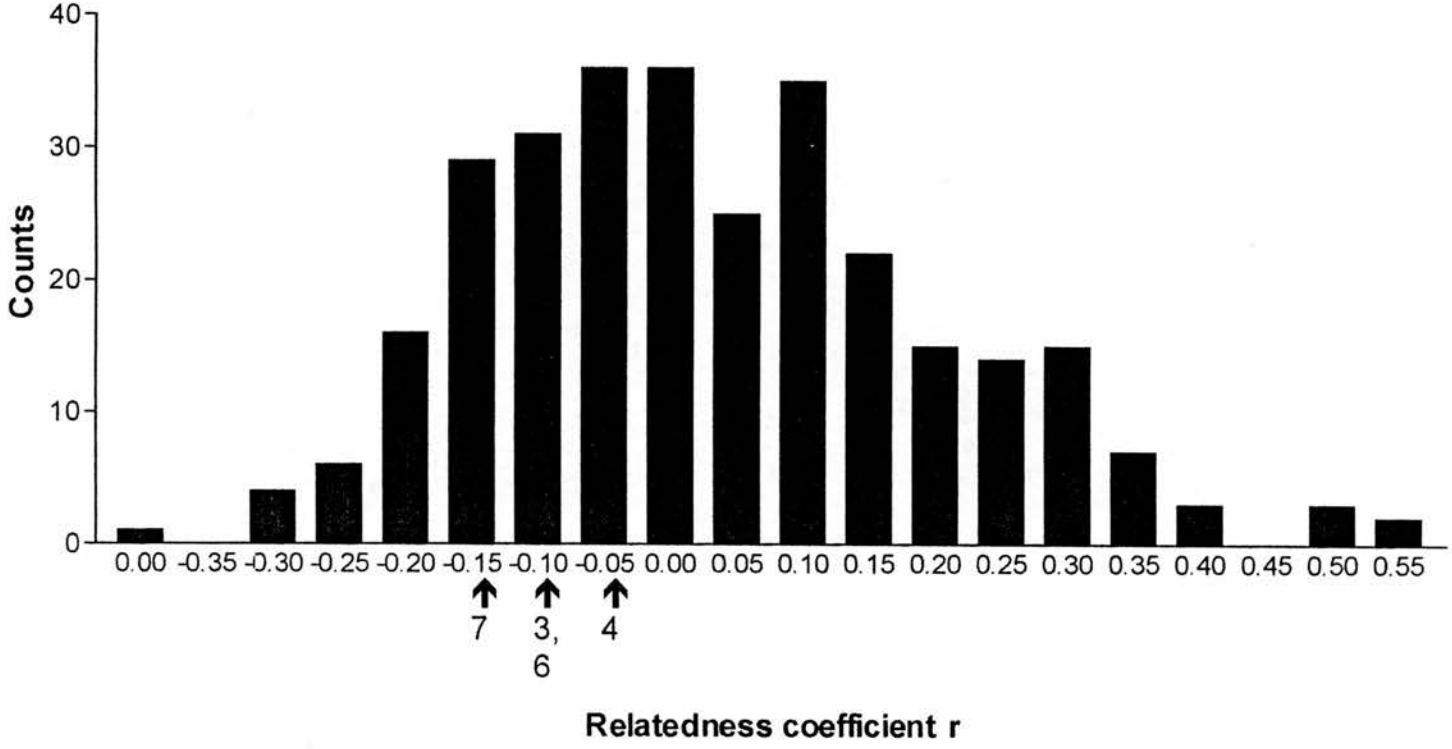


Figure 7.2: Mean pairwise relatedness between major females and recipient chicks

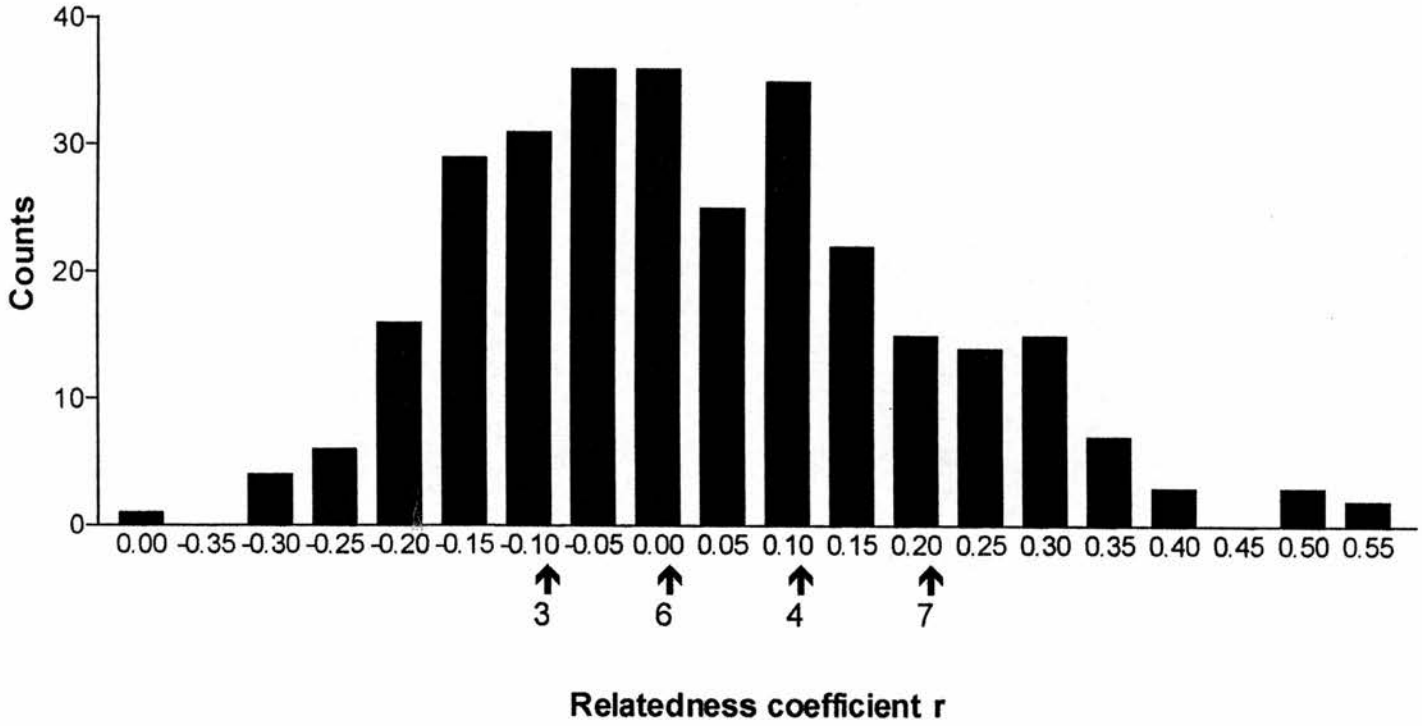


Table 7.1: Bias corrected symmetrical average relatedness coefficient among co-operating females.

	Relatedness coefficient r	Nx, Ny	J/loci	Confidence Interval	Pseudovalue
Nest 3	-0.0204	5, 5	0.0356	0.0842	8
Nest 4	-0.0243	3, 3	0.1206	0.2851	8
Nest 6	-0.0304	4, 4	0.0271	0.0642	8
Nest 7	-0.0750	3, 3	0.0441	0.1043	8
Population	-0.0428	25, 25	0.0006	0.0014	8

Table 7.2: Bias corrected asymmetrical average relatedness coefficient between the major females and recipient chicks.

	Relatedness coefficient r	Nx, Ny	J/loci	Confidence Interval	Pseudovalue
Nest 3	-0.0096	1, 16	0.0806	0.1907	8
Nest 4	0.1287	1, 8	0.1342	0.3175	8
Nest 6	0.0721	1, 8	0.1242	0.2937	8
Nest 7	0.3266	1, 4	0.1951	0.4614	8
Population	-0.0428	25, 25	0.0006	0.0014	8

Relatedness between major females and recipient chicks

Asymmetrical pairwise relatedness was estimated between major females incubating a given nest and the recipient offspring, other than her own. Figure 7.2 illustrates the mean pairwise relatedness which was -0.10, 0.00, 0.10 and 0.20 for nests 3, 6, 4 and 7 respectively.

7.5.2 Average relatedness coefficient

Intra-nest female average relatedness

The average female relatedness coefficient was -0.0204, -0.0243, -0.0304 and -0.0750 for nests 3, 4, 6 and 7 respectively (Table 7.1). The population average relatedness coefficient was -0.0750. The relatedness indices are all less than 0 indicating that the females laying in any given nest are unrelated to each other. This is in agreement with the mean pairwise relatedness estimates discussed above.

Intra-nest major female - recipient offspring relatedness

The relatedness coefficients between the major female and recipient chicks, other than her own offspring, estimated as average

relatedness coefficient was -0.0096, 0.1287, 0.0721 and 0.3266 for nests 4, 6 and 7 respectively (Table 7.2).

7.6 Discussion

Phenotypic traits increasing fitness are expected to be favoured by natural selection. There are exceptions however, where social behaviours lowering fitness, are favoured. This argument can often be preserved by introducing some genetic information, the relatedness of the altruist to the beneficiary even where, as is normally the case, the genetics underlying the particular trait is unknown (Queller 1996). This provides a crucial shortcut for studying social behaviours.

The higher the relatedness of the donor female is to the other recipient females, the less the devaluation of the recipient fitness and therefore the more the likely that expression (1) will be true. This results results in an increase in inclusive fitness favouring selection of the altruistic behaviour. There is a need to estimate the various parameters of (1). The major female lays 8-10 eggs in 16 to 20 days before commencing incubation. There seems to be a constraint to the number of eggs laid by the major female. There may be an increase in predation risk (Hurxthal, 1979) or a significant decrease in hatchability beyond 16 days (Wilson *et al*, 1997; Gonzales *et al*,

1999). The female, however incubates up to 20 eggs in her central clutch while discarding up to 50 eggs onto a peripheral un-incubated clutch. The fitness consequences to her may vary from zero if she were constrained not to lay any more eggs and if incubating the extra eggs was cost free, to the cost of 12 of her own offspring if she were not constrained plus any metabolic, hatchabilty and mortality costs from the extra eggs. In several avian species, incubating extra eggs has been shown to be costly (Thomson *et al* 1998), both in terms of metabolic costs and reduction in hatchability. As earlier mentioned, we were unable to estimate the total fitness consequences in the field. In a case such as this, close relatedness between interacting individuals may be indicative of kin selection favouring the behaviour through an increase in inclusive fitness.

By estimating the ratio of relatedness of altruist to own offspring and to that of recipient chicks as in expression (3), the number of recipient offspring needed to offset genetic loss by the donor can be estimated. The altruistic behaviour will then be favoured.

We therefore set out to investigate if kin selection may be the process driving the apparently altruistic communal nesting system of the ostrich by estimating the relatedness of the interactants. We predicted that the major female is closely related to minor females

that lay in her nest and non-descendant relative of the recipient chicks she cares for.

The mean pairwise relatedness between the major female and the minor females across the four nests were -0.1, -0.05, -0.1and -0.15 for nests 3, 4, 6 and 7 respectively. This was below the median pairwise population relatedness of 0.01 (Figure 7.2). A negative relatedness indicates that the pair is less similar than expected by chance and therefore unrelated.

An average relatedness coefficient estimation also indicated a lack of relatedness among the females across all the nests. This was not different from the population average relatedness coefficient of -0.0428 (Table 1). The major female then, contrary to our prediction, is not a close relative of minor females that lay in her nest. The various females that laid in any of the 4 nests were not related.

Figure 7.2 shows the mean pairwise relatedness between the major females and the recipient chicks. The relatedness showed a large variation from a non-relationship of -0.1 in nest 3, to nearly a half-sib equivalent relationship of 0.2 in nest 7. The average relatedness across the nests was 0.06. Substitution into expression (3) gives a ratio of 8. The major female would then need to incubate 8 times as many recipient chicks for every offspring that she lost, if kin selection was to favour this altruistic behaviour. If the cost to the

major female exceeded 1 egg, this would be unlikely. Clearly then, kin selection would operate if the cost to the ostrich of incubating these extra eggs was very low. This would only be clarified if all the eggs within the clutch were analysed.

The average relatedness coefficient also indicated low levels of relatedness of the major female to the recipient chicks (Table7.2). Even tough the relatedness estimates for nest 7 was 0.33, the confidence level overlapped 0, as did all the other much lower estimates. Again contrary to our prediction, our data show that the major female was not a close non-descendant relative of the recipient chicks.

We thus conclude that kin selection does not seem to drive the apparent altruism in the ostrich communal nesting system. If the costs associated with the incubation of the extra eggs are small or non-existent, incubating extra eggs may be a strategy that counters predation by dilution of offspring of the major female. Indeed, creching is actively initiated by the escorting parents and older chicks. Hurxthal (1979) interpreted this as circumstantial evidence for the anti-predatory hypothesis. A viability decrease would mean that the female would incubate after 8-10 eggs leaving extra space that she may then use to allow other unrelated females access to her

nest and in so doing, receive anti-predatory gains by numerical risk-dilution and predator confusion effects.

Ostriches are long lived and interact in large social groups arising from the creching behaviour. This social group breaks up only when the juveniles reach sexual maturity and begin to breed. Ostensibly, group recognition is possible and since ostriches are multiple breeders, repeated interactions are likely. Direct reciprocity therefore, may be another alternative explanation of the process through which the altruistic communal laying has evolved. Investigations into this would need long-term studies involving known individuals' lifetime or long-term reproductive success and associated behaviour such as females laying in each others nests after lose of a nest or having been major females in an earlier season, as minor females in succeeding seasons.

Traditionally, cooperative breeding has been explained by limited breeding opportunities arising from the species' environment. However it has proved difficult to find any common ecological correlates with cooperative breeding in birds. The life history hypothesis suggests that longevity of a species predisposes it to cooperative breeding. There is evidence for this as birds associated with low mortality and have increased sedentariness. Low latitudes and decreased environmental fluctuations tend to be associated with

cooperatively breeding species (Arnold 1998). This may well be the case in the ostrich which is long lived so that the residual reproductive value remains relatively high in females breeding as minor females in a particular season. Again only long-term measures of reproductive success could verify this.

Chapter 8

FURTHER WORK – ISOLATION AND CHARACTERISATION OF RHEA MICROSATELLITES

8.1 Introduction

There are two rhea species *Pterocnemia pennata* and *Rhea americana*, both native to South America. Rheas are included in the ratite taxa with the ostrich (Cracraft 1974, Sibley & Ahlquist 1990 and Lee *et al.* 1997). The ostrich and the rhea have a similar breeding system with communal laying by several females (Bruning 1974). In the rhea, unlike the ostrich, the parental care is only undertaken by the male. To fully understand the adaptive consequences of the ostrich mating system, it is imperative that phylogenetic holdover be taken into account. If communal nesting is inherited, then the present conditions may not be very informative. A similar study on the breeding systems of other ratites, given the diverse ecological habitats they inhabit, would be useful. I have isolated microsatellite markers for the Darwin's rhea, *P. pennata*, the expectation being that they will be useful in future parentage or population genetic studies.

In Darwin's rhea, incubation and brood care are carried out exclusively by male though the males competes for access to females. Groups of females lay a total of 20-30 eggs in a male's nest, and then apparently move on to lay in several other nests in the same season. Moreover, because the young are relatively small and precocious, male rheas can look after the offspring of several females at once, so that the extensive involvement of males in parental care may not prevent them from being polygynous (Balmford & Barrientos manuscript). Parentage analysis would be useful in finding out if the male's reproductive success correlates positively with his parental investment and effort expended in male-male competition.

8.2 Methods

Samples

Tissues were collected (Balmford & Barrientos manuscript) in the Parque Nacional Torres del Paine, Southern Chile. 58 tissue sample were collected from chicks in 12 nests and preserved in 96% alcohol. Fallen adult feather were collected from the nests and preserved by freezing in-20°C until analysed.

The construction of a genomic library highly enriched for dinucleotide CA and tetranucleotide AAAT repeats

This protocol is adapted from a protocol involving plasmid cloning procedures developed by Olivier Hanotte *et al.* (Department of Zoology, University of Leicester). Microsatellite amplification and screening procedures were as refined by Mike Bruford *et al.* (Institute of Zoology, Regents Park, London) and the enrichment process using biotinylated oligonucleotides was developed by Rob Hammond, Ilik Saccheri and Emma Taylor at the Institute of Zoology (see Armour *et al* 1994). The original protocol from which this protocol is adapted was prepared by Coote (1996). The above methods are adapted into a protocol by Hammond *et al.*(1998).

Restriction digestion

30 μg of pooled DNA from eight individuals was digested with *MbOI* according to the manufacturer's instructions.

DNA restriction fragments ranging from 250-800 bp were recovered and quantified as in chapter 3. Linker sequences

SAULA (20mer 5' GCGGTACCCGGGAAGCTTGG 3') and

SAULB (24mer 5' GATCCCAAGCTTCCCGGGTACCGC 3') were used (Royle *et al* 1992, Armour *et al* 1994). These were annealed using equimolar amounts of each in the presence of 50 mM NaCl at

60°C for 30 minutes. The annealed linkers have a 5' overhang complementary to those generated by the *MboI* restriction digestion. Approximately 2 μg of the annealed linker sequences were ligated at 16 °C overnight, to 200 ng of the size selected DNA using 2 units of T4 ligase enzyme (Pharmacia Biotech®), 1.5 μl 10X buffer and water to a volume of 15 μl. A PCR reaction was then run to amplify the ligated fragments.

Only one strand was covalently bound to the genomic fragment as only the genomic fragment supplies the free 5' phosphate necessary for formation of a diester bond catalysed by T4 ligase. The unbound strand was attached solely by hydrogen complementary binding. The initial extension step of the PCR was to facilitate nick healing by Taq polymerase.

A 25 μl PCR was set up with 1.5 μl (final concentration 1.5 mM MgCl), 2.5 μl 10X buffer, 1.6 μl SAULA primer (25 pMol / μl) 0.8 μl dNTPs (final concentration 10 mM), 2.0 μl ligation reaction, 16.45 μl water and 0.15 μl Taq (0.75 U) (Promega®) in a total volume of 25 μl. The cycling parameters were 1 cycle at 72 °C for 5 minutes to heal the nick followed by 32 cycles of denaturation at 95 °C for 1 minute, annealing at 67 °C for 1 minute and extension at 72 °C for 2 minutes. A final mopping up extension step at 72 °C for 5

minutes was incorporated. 10 µl of the PCR product was then run on a 1.5 % agarose gel along with a negative control of non-PCR ligation at the same dilution (2 µl in 23 µl H_2O) and a 100 bp ladder to check for amplification of the correct size range products. If the PCR was successful, 4 more PCRs were run to provide at least 100 µl reaction that was used in the subsequent hybridisation.

Capture

Biotinylated $(CA)_{22}$ and $(AAAT)_{10}$ oligonucleotides were hybridised with the PCR products to selectively isolate CA and AAAT rich fragments. The biotinylated target molecule has a free 3' end. This is inactivated, in a tailing reaction, by addition of a dideoxynucleotide triphosphate (ddNTP), catalysed by the enzyme Terminal Deoxynucleotidyl Transferase (TDT)(Pharmacia Biotech®). For every 2 pmol of probe used, 0.1 mM ddNTP was used. 40 Units of TDT was used and incubation done at 37 °C for 1-2 hours. The reaction was stopped by 0.1 volume of 0.5 M EDTA.

The PCR product (100 µl) was denatured by boiling for 10 minutes followed by immediated chilling on ice. 5 µg bioltinylated end-tailed $(CA)_{22}$ and $(AAAT)_{10}$ (Gibco BRL®) was then added in separate tubes and made up to 500 µl with 0.5 M sodium

phosphate(Na_2HPO_4) pH 7.4 / 0.5% SDS and hybridisation allowed at 50 °C overnight.

Capture of target molecule – genomic DNA hybrids

50 mg of Vectrex-avidin D resin (Vector laboratories®), and 5 ml of buffer A (150mM NaCl / 100 mM Tris pH 7.5) was added to a screw cap 15ml centrifuge tube and rehydrated in a rotator for 30 minutes at room temperature. Centrifugation for 2 minutes was carried out and the supernatant decanted. The hybridisation solution and 10 ml of buffer A were then added to the avidin pellet. This was gently mixed on an orbital rotator for 30-40 minutes to allow the adherence of the biotinylated probe / genomic DNA hybrids to the avidin. The hybridisation solution was then spun at 3000 rpm for 2 minutes and the supernatant carefully discarded. The avidin resuspended in 10ml buffer A by gentle hand mixing was washed twice more at room temperature.

Specific Washes

Using mini hybridisation oven with gentle agitation, the avidin was washed with 4 ml of 0.1X "buffer A" for 30 minutes at 55°C, centrifuged and the supernatant discarded. This was followed by a 30 minute wash with H_2O, centrifugation and retention of the

supernatant. The supernatant was then concentrated using Centricon-100 (Amicon®) spin columns as earlier described.

Second PCR

Using the concentrated enriched DNA, a second PCR was carried out as before but without the initial extension step.

Removal of SAULA/B linkers

Before cloning, the linkers were removed. Four 25 µl PCR from above were digested with *MboI* as per the manufacture's instructions, concentrated with Centricon-100.On a 1.5 % agarose gel, assessment of the cutting and concentration was done by comparison with uncut genomic DNA containing linker sequences and concentration standards on agarose gel.

20 ng of the enriched DNA fraction was cloned into Pharmacia BAP (dephosphorylated), *BamHI* digested "Ready-to-go" pUC18 vector 100µl Epicurian coli-Blue MR supercompetent cells (Stratagene®) were transformed by heat shock according to the manufacturer's instructions.

Genomic library

The entire batch of transformed cells was split into 4 aliquots adding 260 µl of Xgal (5.2 mg) and 26 µl IPTG (5.2 mg) as selective markers (Chapter 3). These were plated out in Luria Broth(LB)/ tetracycline (12.5µg / ml)/ampicillin (50µg / ml) and incubated for 16 h at 37 °C. Individual white colonies were tooth-picked to 0.5 ml tubes of 400 µl LB/tetracycline / ampicillin as above. These were incubated with shaking at 37 °C for 16 h.

2 µl of the overnight culture was plated onto LB / antibiotic agar plates with a grid acetate background for colony identification. These were incubated for a further 16 h at 37 °C. The Southern blotting, radiolabel probing, hybridisation, autoradiography, small scale isolation of plasmid DNA, sequencing of the insert, designing the primers and screening of the microsatellite primers were carried out as detailed in chapter 3.

8.3 Results

Out of 290 colonies screened 48 positives colonies were detected. 21 were selected for sequencing on the basis of signal intensity. Of these 21, five had obscure reverse sequences which subsequently did not align, one had no flanking sequence

downstream, 3 had ambiguous sequence and were therefore not useful and 12 gave useful sequences with repeat motifs with greater than 20 bp length and sufficient flanking regions for primer design. Primers were designed for these 12 loci and PCR products tested for polymorphism.Eight of the sequences had PCR products that were monomorphic or had extensive PCR slippage resulting in poor resolution. Only four microsatellite loci provided good resolution and were polymorphic. These were PTP03 (EMBL accession no. AF230714), PTP05 (EMBL accession no. AF230715), PTP06 (EMBL accession no. AF230716) and PTP08 (EMBL accession no. AF230717). The observed heterozygosity ranged from 0.58 to 1.00 (Table 8.1).

The PCR cycling conditions were as Chapter 3 but with the specified annealing temperatures for each locus. The cocktail reaction was as used in chapter 3 and in all reactions a final $MgCl_2$ concentration of 1.5 mM was used.

Table 8.1

Characterisation of four microsatellite loci in the rhea *Pterocnemia pennata*. The number of alleles was obtained for 12 unrelated individuals.

Locus	length of PCR product	Primer sequence (5' - 3')	Repeat motif	Annealing temp.(^{O}C)	No. of alleles	H_O
PTP03	216	F: CCACCAGCCTTGAGTTTACC R: TGCATCTCAGGTTTCATGTTT	$(CA)_{11}$	55	14	0.83
PTP05	181	F: CTCGTTTTTCCTGCAACACA R: AGTCCTTTCCACCTCAACCA	$(CA)_7CTTG(CA)_7$	55	6	1.00
PTP06	208	F: GGCACTCTCATTTGCAGGTT R: AAAGGGATGCAGCTGTCTGT	$(CA)_{17}(GA)_6$	56	12	0.67
PTP08	193	F: TCAATATGGTGAAATGGCACA R: TATTCAAAAGGCCACCTTGC	$(CA)_{11}$	53	3	0.58

A preliminary examination of two rhea nests indicated that there was multiple parentage in one of them (Table 8.2). Since we were unable to extract DNA from the dropped adult (presumably male) feathers that were on the nest, we could not carry out paternity analysis.

It is hoped that the rhea microsatellites isolated here will inspire similar investigations into ratite breeding system and in so

doing enable us gain better understanding of the evolution of ratite breeding behaviour.

Table 8.2 Genotypes of chicks in two Rhea (Pterocnemia americana) nests, nests 2 and 9 (Balmford manuscript) respectively. In nest 2, locus PTP03 and PTP06 scored > 4 alleles indicating the occurrence of multiple parentage. Similarly, PTP05 scored > 4 alleles in nest 9. Al. denotes allele.

Nest	Chick	PTP03		PTP05		PTP06		PTP08	
		Al.1	Al.2	Al.1	Al.2	Al.1	Al.2	Al.1	Al.2
2	1	242	214	186	186	210	204	186	186
	2	246	236	194	186	220	214	196	186
	3	236	230	186	186	226	214	198	186
	4	248	214	194	186	226	214	192	186
	5	238	214	194	186	214	206	198	186
	7	238	214	194	186	214	206	186	186
	8	238	230	194	186	226	214	198	196
	9	248	230	194	186	214	206	186	186
	10	220	214	186	186	214	206	198	186
	11	248	220	186	186	214	206	198	192
	12	230	214	186	186	214	206	186	186
	13	230	214	194	186	214	206	186	186
	14	230	214	186	186	214	206	186	186
9	1	216	216	186	184	228	208	228	208
	2	224	216	194	184	208	208	208	208
	3	218	216	196	194	200	208	200	208
	4	216	216	204	184	208	208	208	208
	8	216	216	194	184	200	206	200	206
	11	216	216	184	184	208	208	208	208

Chapter 9

GENERAL DISCUSSION AND CONCLUSION

9.1 General discussion

The study of mating systems, often in the context of how they affect reproductive success (Emlen & Oring 1977, Davies 1991) focuses on ways individuals obtain mates, the number of individuals they mate with, how long they stay together and the allocation of parental care. Mating systems have often been described from behavioural data alone but molecular techniques can reveal unexpected patterns of gene transmission (pedigree connection) resulting from diverse behavioural tactics that individuals employ (Hughes 1998). Here I used molecular microsatellite markers (Kimwele *et al.* 1998) to analyse parentage in the complex ostrich breeding system comprising polygynandrous mating, communal nesting and biparental care system (Sauer & Sauer 1966, Hurxthal 1979, Bertram 1982)

Paleognathiformes, comprising ratites and tinamous show prominent or exclusive paternal care of eggs and offspring. This unusual parental care pattern is associated with a diverse array of mating systems ranging from monogamy to promiscuity (Handford &

Mares). Paternal care, mixed polygyny/polyandry and communal egg laying is unusual but typical of rheas (Bruning 1974). The ostrich has a similar breeding system that differs primarily in that the female also provides parental care. Communal nesting is also found in certain tinamous species (Handford & Mares 1985). Since the tinamous are an outgroup to the ratites (Cracraft 1974, Sibley & Ahlquist 1990, Cooper, 1997) and the rhea and ostriches have been placed in separate sister groups (Lee 1997, Cooper 1997), it is likely that communal nesting evolved independently. However all paleognaths, with the exception of the ostrich, have a paternal care system. The most parsimonious explanation to the biparental ostrich care is that it is a derived condition. Biparental care may be a response to ecological factors such as increased predation or decreased hatchability as a result of diurnal temperature fluctuations experienced in the savanna habitat. Such factors would require 24 h care of eggs and a single parent could not succeed. In farm storage conditions hatchability has been found to decrease after 10 days (Gonzalez *et al.* 1999). There are numerous ungulates and large predators in tropical Africa that increase the risk of nest destruction. This may set up selective pressure leading to biparental care.

The ostrich has an unusual breeding system in birds. The mating system has been termed variously as serially polygynous or

polgynandrous (Sauer & Sauer 1966, Hurxthal 1979, Bertram 1979). The breeding behaviour however has monogamous aspects with a pair bond between the territorial male and the major hen. Hurxthal (1979) termed it "facultatively polygynous".

The major females and the territorial males seek extra pair matings. All the females and males analysed in this study engaged in extra pair copulations. Out of 59 eggs assigned parentage, 42 were extra pair eggs, an extremely high 71.2%. This is one of the highest reported extra pair fertilisation rate in birds. For example, the superb fairy wren has the highest known extra pair paternity rate of 76% (Mulder *et al.* 1994). 2.4% has been documented in in zebra finches *Taenopygia guttata* (Birkhead *et al.* 1990), 13.6%. in house sparrows *Passer domesticus*, (Wetton and Parkin 1991), 14 - 18% in shags *Phalacrocorax aristotelis* (Graves *et al.*1992), 11% in blue tits *Parus caeruleus* (Kempenaers *et al.* 1992), and 55% in reed buntings *Emberiza schoeniclus* (Dixon *et al.* 1994)..

Here I found that Intra specific brood parasitism(IBP), the laying of eggs in conspecific nests, was a common reproductive strategy employed by the female ostrich. IBP is likely in species such as the ostrich that have limited nesting opportunities, are unable to effectively guard their nests, have precocial chicks that need minimal

parental investments and are long lived and therefore have a high residual reproductive value (Yom-Tov 1980)

Why do the major females engage in extra pair copulations? Sperm storage occurs in birds (Birkhead & Moller 1992) and has been demonstrated in ostriches (Swain & Sicouri 1999) thus one insemination is likely to fertilise an entire clutch (Birkhead & Moller 1992). Also, the cost to females of engaging in extra-pair copulation may be high as a result of physical male aggression (Zenone *et al.* 1979) or restricted paternal care (Burke *et al* 1989, Dixon *et al* 1994). In birds, the evidence indicates that they may do so to gain indirect genetic benefits from high quality males (Kempenears *et al.* 1992), as an assessment of future mates (Ens *et al.* 1993) or to gain from paternal care (Davies *et al.* 1996). Since major and minor females range freely in a large home range covering several males territories, and males solicit for matings which are commonly accepted but may be rejected (Hurxthal 1979), harassment seems an unlikely explanation. The high number of males mating with females and the fact that a single insemination is likely to be all that is required to fertilise a clutch makes the fertility insurance hypothesis also improbable. There is also no indication that the females may be benefiting from indirect genetic benefits. Hurxthal (1979) found that the territorial males were neither dominant nor larger than non-

territory holders. It may be that females seek extra pair copulations to assess future mates. This hypothesis can be tested in the future by assessing the mating patterns of known individuals over several seasons.

That the territorial males seek extra pair copulation is not surprising. Since male gametes are numerous and cheap, and the territorial male is unable to guard his mate as she ranges outside his territory, he can attempt to maximise his reproductive success by mating repeatedly with his mate while provisioning for their offspring and engaging in extra pair copulation to sire additional offspring, that he may or may not provision for (Trivers 1972). Hurxthal (1979) observed repeated copulation but could not ascertain wether these were intra or extra pair copulations. The fact that only one intra pair egg was laid outside the pairs nest indicates the possibility that the male employs frequent intra pair copulation as a paternity guard. This is an area that could be investigated in the future. The territorial male and major female also provide parental care to other females' eggs that exploit the extra space available in the nest arising as a result of the major female constraint to lay between 7-11 eggs (Hurxthal 1979, Betram 1982). However, I found that the territorial male and major female incubate an average of 23 eggs (Hurxthal (1979) and Bertram (1982) found that 20-21 eggs

were incubated). Because the male mates preferentially with any new female entering his territory, he is likely to have a genetic interest, not just in eggs laid by his major female, but also in those laid by the minor female. In this study, I have shown that the territorial male fertilises, with the same success rate, both the major and minor females' eggs.

There is circumstantial evidence that the pair bond is unlikely to be long term since all the females in the study mated with other males and laid eggs, most likely earlier on in the breeding season, in other nests prior to becoming a major female with a particular territorial male. Unfortunately, my field observations were limited and I identified the nests only during incubation. I was unable to establish the chronological order that the females laid in various nests or that the nests were established. This, however, is discussed below. Nest 3 was an unusual nest, the major female only laid had two eggs in the central clutch though she laid three eggs in nest 6 and five in nest 4. There is a possibility that she took over from another major female for an unknown reason or was unable to select her eggs. Because of the large number of eggs she laid elsewhere, it is likely that she was a minor female who took over the nest.

"The temporal nest distribution hypothesis" may offer an alternative explanation to the apparently altruistic ostrich communal laying. Temporal nest distribution may result in differential nest availability thereby leading to communal laying; a consequence of several females laying in scarcer early nests as minor females before starting their own nests later on in the season. Field studies have identified IBP by identifying abnormalities in sequence of egg laying or phenotypic differences in eggs (Moller 1989, Brown &Brown 1989, Bertram 1992, McRae & Burke 1996). I was unable to directly monitor this in the field, however, parentage analysis done here provides circumstantial evidence of the temporal sequence of female laying in the various nests sampled. If it is assumed that Nest 3's female took over the nest at or near completion, nest 3 was likely to be the earliest nest among those analysed since all but one of the sampled females contributed to the clutch. A maximum of 7 females laid in nest 3 (figure 5.7). Nest 7 may have to been the next nest. This is likely because Nest 6's female laid in this nest despite the fact that six minor females, the second most numerous, laid in her own nest (table 5.9). Nest 7, with a maximum of 4 laying females (table 5.10), may have had less than the expected number of females laying in it (6-7) as it was isolated by distance and a forest barrier. Nest 6, 4 and 8 may have followed respectively with a maximum of 6, 2 and an

undefined number of females laying in these nests respectively (tables 5.8, 5.9). Nest 8 is known to be the youngest as it was discovered at the beginning of egg laying when all other nests were at the start of incubation. Again with the exception of nest 3 female, the number of eggs laid by the major females in other nests supports this hypothesis. Nest 7 (probably the second nest) female lay only 2 eggs in the presumable earliest nest 3 before starting her own nest; nest 6 female (probably the third nest) lay 4 in the earliest nest (nest 3) and 1 in nest 7 (which was closer despite the forest barrier) before starting her own nest; Nest 4 female lay an egg each in earlier nests 7 and 6 before beginning her own nest. Finally the female in nest 8 which was known to be the youngest, lay 10 eggs, the highest incidence of egg dumping, in other available nests before laying in her own nest (figure 5.2). The fact that all but one intra pair egg were laid in the pair nest support the assertion that the females all lay initially as minor females before starting their own nests as major females. This hypothesis, however, needs further collaborative data from field studies. Further data should, preferably, be collected from populations that have no sex ratio skews arising for example arising from a high population density and therefore scarce nesting sites.

Since only territorial males mate, I found an operational sex ratio skew of approximately 1:3 in favour of females (Chapter 3). The

males I was able to sample were all territorial males. I was unable to collect and analyse reproductive success data from a large random sample of mature males in the park, including those that did not gain territories. Since non-territorial males may get some as yet unmeasured copulation (Hurxthal 1979) this would have been useful for detecting any variance in reproductive success among the males in breeding condition. It is hoped that such data will be collected in the future.

Presumably female ostriches can increase their reproductive success by becoming a major female and co-operating with the male in a pair bond to provide parental care in the form of egg guarding, incubation, brooding and chick escorting. They also seek reproductive success in conflict with their mate, the male seeking to mate with other females who may or may not lay his eggs in his nest thereby parasitising not only his major female's reproductive effort but also other individuals. The major female likewise mates with other males and as a minor female, lays in their nests.

The male mates with multiple minor females that enter his territory and probably lay in his nest. The major female also has extra space that she allows to be exploited by these other minor females. It may be that she is unable to stop other females laying due to a high physical cost of a conflict e.g. injury, damage to eggs and predation

or due to the physical disadvantage of a sitting bird (Bertram 1982). This is unlikely as the female readily gets up to give way to an incoming female. A possible benefit may arise from a dilution effect of the eggs and subsequently the chicks (Hurxthal 1979). This hypothesis has not yet been tested. The major female may also gain by giving access to relatives to lay in her nest. I found no evidence that the females laying in a communal nest were close relatives. Kin selection seems unlikely to be responsible for this apparently altruistic female behaviour. Since most of the females studied here lay in each others nest as minor females, it is possible that due to the large social grouping evident (mean of 12 adults outside the breeding season (Hurxthal 1979)), major females may be reciprocating to accompanying (or non accompanying) minor females that previously allowed them laying access or may give them future access to their nests. This hypothesis is yet to be tested.

This study illustrates the potential of molecular techniques in helping us understand the behaviour and other ecological aspects of animal species that are difficult to observe either due to time, costs or other limitations. I was unable to mark the birds under study due to National Park regulations nor monitor them for prolonged periods of time. The ostriches are physically imposing yet very shy about revealing where there nest is as would be expected of ground nesters

with numerous predators. Therefore, they are difficult to observe at the nest. Despite this, I was able to analyse tissue collected with no invasiveness at the nest. Had the chicks hatched, plucked feathers would have been adequate to carry out my analysis. I was then able to use the microsatellite genetic markers to amplify specific loci in the *S..c. massaicus* breeding population at the Nairobi National Park. I used this information to gain an understanding of the genetic basis of the ostrich mating system.

9.2 Conclusion

This study revealed high levels of extra pair fertilisations exhibited by both the major female and the territorial male in the ostrich communal nesting system. Laying in other nests as a minor female was also common among females that later became major females. Though not conclusively demonstrated, the major female may be able to select her own eggs for retention and incubation in the central clutch. She was also able to combine the minor and major female strategies and in so doing attempted to maximise her reproductive success. The territorial male mated with both his major female and any other minor female entering his territory thereby also maximising his reproductive success. The pair, though employing conflicting strategies, also achieved substantial intra pair fertilisation;

they also complemented each other by providing parental investment and in so doing ensured that both their reproductive success was maximised.

9.3 Future work

A future study would be to monitor and collect samples from a large number of individuals within a population at the southern migratory corridor of Nairobi National Park for at least three breeding seasons. This is an area that is outside the direct jurisdiction of the Kenya Wildlife Services and tourist interference is minimal. Therefore there would be limited restrictions on safe marking for individual identification. Such a study would involve collection of feather samples from hatched chicks and all discarded eggs from the peripheral clutches for incubation and hatching at commercial hatcheries such as the Maasai Ostrich farm, Kitengela. Parentage analysis using the same suite of microsatellite markers used here would estimate reproductive success of major and minor females along with those of territorial and non-territorial males.

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Appendix I

Population genotyped with eight microsatellites OSM1,2,4,5,6,7 and List005 and List 009.

ID	osm1al1	osm1al2	0sm2al1	osm2al2	osm4al1	osm4al2	osm5al1	osm5al2	osm6al1	osm6al2	osm7al1	osm7al2	kemp5al1	kemp5al2	kemp9al1	kemp9al2
N6-c1	113	93	147	143	136	134	200	178	0	0	186	186	207	199	288	282
N6-c2	119	113	147	143	138	136	200	172	0	0	188	188	213	193	288	288
N6-c3	117	113	147	143	134	132	196	182	0	0	186	186	199	199	306	282
N6-c4	123	119	153	143	134	132	182	182	0	0	210	186	195	193	288	276
N6-c5	119	113	147	143	134	132	182	182	0	0	186	186	207	193	288	282
N6-c6	119	113	155	143	136	134	196	182	0	0	186	186	207	199	312	306
N6-c7	113	93	147	143	136	134	202	178	0	0	0	0	209	199	312	288
N6-c8	113	93	143	143	136	134	202	182	0	0	218	188	209	207	282	266
N6-c9	119	113	155	143	136	134	0	0	0	0	188	188	207	199	312	282
N6-c10	119	119	153	143	138	136	200	178	0	0	180	180	199	193	374	288
N6-c11	123	97	153	145	148	134	178	178	0	0	216	186	207	199	330	266
N6-c12	113	113	149	143	148	136	0	0	0	0	188	188	207	199	330	266
N6-c13	119	113	155	147	136	134	196	172	0	0	186	186	199	199	330	282
N6-c14	117	113	155	143	136	134	178	172	0	0	188	188	207	199	330	282
N6-c15	117	113	147	143	136	134	196	172	0	0	188	188	209	199	288	282
N6-c16	129	119	143	143	136	134	202	178	0	0	186	186	195	195	330	312
N6-c17	119	113	147	143	134	132	196	182	0	0	188	188	199	193	308	278
N6-c18	119	93	180	143	138	136	182	182	0	0	216	188	213	193	281	266
N7-c1	113	113	159	159	136	136	196	182	111	105	186	186	207	193	330	287
N7-c2	119	115	159	159	140	140	182	182	111	111	219	180	209	199	330	287
N7-c3	113	113	159	159	150	136	202	178	107	103	219	186	213	199	265	265
N7-c4	113	113	163	159	150	136	202	196	111	103	219	219	199	193	330	283
N7-c5	115	115	159	159	136	136	182	178	111	103	219	219	199	193	330	283
N7-c6	127	113	159	159	140	140	202	178	109	103	217	180	213	193	330	278

N7-c7	130	113	159	159	138	138	196	178	111	111	186
	180	199	193	330	330						
N7-c8	113	113	159	159	136	136	182	182	111	105	186
	186	207	193	287	287						
N7-c9	113	113	159	159	140	140	182	178	111	111	186
	180	193	193	303	287						
N7-c10	0	0	159	159	138	138	182	182	111	111	217
	217	207	199	287	287						
N7-c11	129	113	159	159	150	138	196	182	111	105	217
	217	207	199	287	287						
N7-c12	113	113	159	159	140	140	196	182	111	105	217
	186	199	193	330	303						
N3-c1	121	121	147	143	0	0	184	184	104	104	188
	156	199	199	290	266						
N3-c2	113	99	155	143	0	0	196	196	110	110	188
	156	199	199	332	332						
N3-c3	113	113	151	143	0	0	202	182	110	106	156
	156	199	193	290	266						
N3-c4	113	113	180	155	0	0	178	172	104	104	186
	186	213	199	314	266						
N3-c5	131	121	147	147	0	0	200	178	110	110	190
	156	0	0	290	290						
N3-c6	123	113	180	157	0	0	182	178	106	106	186
	156	207	199	290	290						
N3-c7	113	113	147	143	0	0	178	178	110	104	156
	150	199	193	332	314						
N3-c8	129	113	149	143	0	0	196	196	110	104	186
	158	199	195	332	266						
N3-c9	113	113	180	143	0	0	202	182	110	110	156
	150	207	193	290	290						
N3-c10	131	113	157	147	0	0	178	178	110	110	188
	156	199	199	278	278						
N3-c11	113	113	147	143	0	0	182	178	110	102	190
	190	199	199	290	284						
N3-c12	127	113	153	143	0	0	178	172	104	104	180
	158	207	199	290	266						
N3-c13	123	113	180	155	0	0	182	178	110	106	186
	156	199	199	332	290						
N3-c14	113	113	149	143	0	0	206	184	110	110	158
	158	213	195	332	266						
N3-c15	113	99	149	149	0	0	202	196	0	0	188
	156	199	199	332	284						
N3-c16	126	113	160	143	0	0	206	178	110	110	156
	156	199	195	304	266						
N3-c17	127	113	143	143	0	0	202	172	104	104	186
	158	213	207	290	266						
N3-c18	127	113	143	143	0	0	202	172	104	104	186
	158	213	207	290	266						
N4-c1	119	119	153	143	139	139	178	178	112	106	190
	188	207	193	284	276						
N4-c2	127	113	160	147	141	141	200	182	108	106	188
	188	207	199	284	262						
N4-c3	123	119	143	143	150	139	202	178	112	108	188
	188	207	199	284	262						
N4-c4	123	119	149	149	150	139	202	178	112	108	188
	188	207	199	284	284						
N4-c5	127	113	160	153	139	139	200	178	108	108	188
	188	207	199	280	262						
N4-c6	113	113	160	149	139	139	200	182	108	106	188
	188	207	199	280	262						

N4-c7	123	119	149	149	150	139	202	178	112	108	188
	188	207	199	284	262						
N4-c8	127	117	147	147	141	141	196	172	106	106	190
	188	207	199	330	312						
N4-c9	127	119	153	147	150	141	182	182	108	106	188
	188	207	199	330	280						
N4-c10	127	119	180	147	139	139	182	172	108	106	188
	188	207	193	298	284						
N4-c11	127	119	180	147	139	139	182	172	108	106	188
	188	207	193	298	284						
N4-c12	123	113	153	147	150	139	184	184	108	106	188
	188	213	199	280	262						
N4-c13	123	113	180	153	150	139	178	178	112	108	190
	188	199	195	330	272						
N6male	119	119	155	147	136	134	182	172	0	0	188
	188	207	199	312	288						
N6fem	113	93	155	143	136	134	196	178	0	0	188
	186	199	199	330	306						
N7male	113	113	159	159	136	136	196	182	110	106	186
	186	199	193	330	303						
N7fem	127	113	159	159	140	140	196	178	110	104	217
	180	213	193	330	287						
N3male	119	113	149	143	0	0	202	196	110	104	186
	180	209	199	290	266						
N3fem	119	99	147	143	0	0	196	172	110	102	188
	188	332	318	199	199						
N4male	113	113	160	143	139	139	200	182	112	108	190
	188	213	207	284	260						
N4fem	127	113	149	149	141	141	182	182	112	106	188
	188	209	199	284	262						
1M	131	113	147	147	137	135	204	182	104	104	212
	190	213	213	312	288						
2M	113	113	155	155	141	135	182	178	110	102	212
	188	213	209	312	282						
3M	115	105	149	149	139	137	182	178	110	104	188
	182	209	209	213	288						
4M	127	113	149	149	149	135	182	178	106	102	188
	188	199	199	332	268						
5M	115	105	180	147	135	135	180	178	110	106	188
	182	213	209	282	282						
6M	113	105	155	143	137	137	198	182	110	104	188
	188	207	195	302	286						
7M	119	109	153	143	137	135	178	178	110	110	190
	190	199	195	332	268						
1F	131	111	0	0	135	135	178	178	106	104	220
	220	199	199	350	302						
2F	113	105	153	149	149	139	182	178	110	104	212
	188	199	199	332	288						
3F	127	113	153	149	149	137	182	182	106	112	218
	188	209	193	332	284						
4F	127	113	153	147	137	137	182	172	106	104	190
	182	199	199	314	304						
5F	127	113	147	143	135	135	178	178	110	106	220
	182	199	193	288	268						
6F	117	105	155	149	139	137	198	178	104	104	212
	188	209	199	350	284						
7F	113	105	180	153	135	135	208	178	106	106	212
	190	207	199	332	332						
8F	131	121	155	149	149	139	182	178	104	102	212
	190	207	193	288	284						

N2f	113	113	149	143	149	135	204	182	106	104	190
	182	199	193	288	284						
F8	119	113	180	160	139	135	178	178	110	104	218
	188	210	210	350	278						

Appendix 2

Parentage analysis output

1) Nest 3Territorial Male

**** Loci ****

1 osm1
2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Summary statistics ****

Offspring: 18
Tested: 18

Known parent typed at 4 or more loci: 0
Known parent typed at fewer than 4 loci: 18
Not tested: 0
Candidate parents (total): 4
Candidates sampled: 4 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 4 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	13 (18)	72% (100%)	
Relaxed	80.00	0.00	13 (18)	72% (100%)	
Parentage unresolved			5 (0)	28% (0%)	

Major female Nest 3

**** Loci ****

1 osm1
2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp5
8 kemp9

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Summary statistics ****

Offspring: 18
Tested: 18
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 18
Not tested: 0
Candidate parents (total): 6
Candidates sampled: 6 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00		16 (18)	89% (100%)
Relaxed	80.00	0.00		16 (18)	89% (100%)
Parentage unresolved				2 (0)	11% (0%)

Nest 4 territorial male

**** Loci ****

1 osm1
2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10

Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Summary statistics ****

Offspring: 13
Tested: 13
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 13
Not tested: 0
Candidate parents (total): 4
Candidates sampled: 4 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	12 (13)	92% (100%)	
Relaxed	80.00	0.00	12 (13)	92% (100%)	
Parentage unresolved			1 (0)	8% (0%)	

Nest 4 major female

**** Loci ****

1 osm1
2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Summary statistics ****

Offspring: 13
Tested: 13
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 13
Not tested: 0
Candidate parents (total): 6
Candidates sampled: 6 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	13 (13)	100% (100%)	
Relaxed	80.00	0.00	13 (13)	100% (100%)	
Parentage unresolved			0 (0)	0% (0%)	

Nest 6 Territorial male

**** Loci ****

1 osm1
2 osm2
3 osm4

4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Missing genetic data ****

Offspring IDs not found in the genotype file:

OSM1AL1

TOTAL: 1

**** Summary statistics ****

Offspring: 19
Tested: 18
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 18
Not tested: 1
Candidate parents (total): 4
Candidates sampled: 4 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	14 (18)	78% (100%)	
Relaxed	80.00	0.00	14 (18)	78% (100%)	
Parentage unresolved			4 (0)	22% (0%)	

Nest 6 major female

**** Loci ****

1 osm1
2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Missing genetic data ****

Offspring IDs not found in the genotype file:

OSM1AL1

TOTAL: 1

**** Summary statistics ****

Offspring: 19
Tested: 18
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 18
Not tested: 1
Candidate parents (total): 4
Candidates sampled: 4 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	14 (18)	78% (100%)	
Relaxed	80.00	0.00	14 (18)	78% (100%)	
Parentage unresolved			4 (0)	22% (0%)	

Nest 7 Territorial male

**** Loci ****

1 osm1
2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000

Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Missing genetic data ****

Offspring IDs not found in the genotype file:

OSM1AL1

TOTAL: 1

**** Summary statistics ****

Offspring: 13
Tested: 12
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 12
Not tested: 1
Candidate parents (total): 4
Candidates sampled: 4 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	10 (12)	83% (100%)	
Relaxed	80.00	0.00	10 (12)	83% (100%)	
Parentage unresolved			2 (0)	17% (0%)	

Nest 7 major female

**** Loci ****

1 osm1

2 osm2
3 osm4
4 osm5
5 osm6
6 osm7
7 kemp005
8 kemp009

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Missing genetic data ****

Offspring IDs not found in the genotype file:

OSM1AL1

TOTAL: 1

**** Summary statistics ****

Offspring: 13
Tested: 12
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 12
Not tested: 1
Candidate parents (total): 6
Candidates sampled: 6 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.00	10 (12)	83% (100%)	
Relaxed	80.00	0.00	10 (12)	83% (100%)	
Parentage unresolved			2 (0)	17% (0%)	

Massai ostrich farm – male parentage

**** Loci ****

1 osm1
2 osm2
3 osm3
4 osm4
5 osm7

**** Parameters ****

Input
 Cycles: 1000
 Number of candidate parents: 10
 Proportion of candidate parents sampled: 1.000
 Proportion of loci typed: 1.000
 Proportion of loci mistyped: 0.010
Output
 Relaxed confidence level: 80.00%
 Strict confidence level: 95.00%

**** Summary statistics ****

Offspring: 9
 Tested: 9
  Known parent typed at 1 or more loci: 0
  Known parent typed at fewer than 1 loci: 9
 Not tested: 0
Candidate parents (total): 2
 Candidates sampled: 2 (100%)

Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.79	4 (6)	44% (65%)	
Relaxed	80.00	0.00	9 (9)	100% (100%)	
Parentage unresolved			0 (0)	0% (0%)	

Massai ostrich farm – female parentage

**** Loci ****

1 osm1
2 osm2
3 osm3
4 osm4
5 osm7

**** Parameters ****

Input
Cycles: 1000
Number of candidate parents: 10
Proportion of candidate parents sampled: 1.000
Proportion of loci typed: 1.000
Proportion of loci mistyped: 0.010
Output
Relaxed confidence level: 80.00%
Strict confidence level: 95.00%

**** Summary statistics ****

Offspring: 9
Tested: 9
Known parent typed at 1 or more loci: 0
Known parent typed at fewer than 1 loci: 9
Not tested: 0

Candidate parents (total): 4
Candidates sampled: 4 (100%)
Candidates not sampled: 0 (0%)
(0 sampled candidate parents typed at fewer than 1 loci were excluded from analysis)

Neither parent known

Confidence	Level(%)	Delta	Criterion	Tests	Percentage
Strict	95.00	0.79	5 (6)	56% (65%)	
Relaxed	80.00	0.00	9 (9)	100% (100%)	
Parentage unresolved			0 (0)	0% (0%)	

****
****

APPENDIX 3 Adult population pairwise relatedness coefficients.

	N6m	N6f	N7m	N7f	N3f	N3m	N4m	N4f	1m	2m	3m	4m	5m	6m	7m	1f	2f	3f	4f	5f	6f	7f	8f	N2f	F8
N6m	*																								
N6f	0.08	*																							
N7m	-0.17	0.23	*																						
N7f	-0.38	-0.14	0.25	*																					
N3f	0.29	0.00	-0.17	-0.11	*																				
N3m	-0.23	0.00	0.06	0.00	-0.05	*																			
N4m	-0.14	-0.20	-0.10	-0.16	-0.14	-0.22	*																		
N4f	0.08	-0.09	-0.03	-0.20	-0.05	-0.04	0.14	*																	
1m	0.03	-0.29	-0.13	0.05	-0.10	-0.10	0.08	-0.08	*																
2m	0.05	0.02	-0.01	0.01	-0.03	-0.09	0.11	0.22	0.23	*															
3m	-0.04	-0.24	-0.21	-0.07	-0.05	0.13	-0.05	0.31	0.09	0.11	*														
4m	0.08	0.12	-0.05	-0.16	-0.01	-0.06	-0.07	0.53	-0.10	0.16	0.19	*													
5m	-0.09	-0.22	-0.19	-0.06	0.03	-0.17	-0.16	-0.02	0.16	0.31	0.28	0.08	*												
6m	0.13	-0.06	-0.13	-0.12	0.11	-0.09	0.11	0.10	0.11	0.21	0.24	0.03	0.00	*											
7m	-0.13	-0.04	-0.16	-0.04	-0.01	0.02	-0.13	-0.22	0.01	0.02	0.06	0.10	0.13	0.09	*										
1f	-0.17	0.06	-0.14	-0.07	-0.23	-0.10	-0.29	-0.09	0.08	-0.01	-0.03	0.24	0.21	-0.13	0.23	*									
2f	0.01	0.06	-0.05	-0.11	-0.16	0.03	0.00	0.20	0.08	0.11	0.37	0.49	-0.04	0.11	0.19	0.14	*								
3f	-0.07	-0.29	0.02	-0.10	-0.15	-0.15	0.14	0.56	0.02	0.10	0.28	0.39	-0.04	0.17	-0.08	-0.17	0.22	*							
4f	-0.02	-0.06	0.00	-0.12	-0.17	-0.09	-0.09	0.10	0.29	-0.12	0.05	0.16	-0.06	0.14	0.15	0.11	0.19	0.24	*						
5f	-0.18	0.00	-0.02	0.07	-0.11	-0.10	-0.18	-0.09	0.10	0.13	0.04	0.28	0.37	-0.14	0.38	0.52	0.10	-0.05	0.13	*					
6f	-0.06	-0.02	-0.30	-0.09	-0.14	0.04	-0.06	0.14	0.15	0.11	0.48	0.13	0.04	0.32	-0.01	0.20	0.39	0.07	0.11	-0.11	*				
7f	-0.19	-0.10	-0.04	-0.14	-0.25	-0.19	-0.06	-0.05	0.07	0.10	-0.10	0.29	0.31	-0.08	0.33	0.34	0.26	0.12	0.16	0.33	0.01	*			
8f	-0.04	-0.27	-0.22	-0.07	-0.20	-0.21	0.11	0.03	0.23	0.14	0.25	0.09	-0.15	0.00	0.01	0.04	0.27	0.17	-0.07	-0.02	0.33	0.03	*		
N2f	-0.20	-0.09	0.12	-0.07	-0.27	0.07	0.18	0.23	0.30	0.07	0.11	0.30	-0.01	0.00	0.01	0.10	0.27	0.32	0.28	0.35	0.03	0.22	0.29	*	
F8	-0.08	-0.13	-0.18	0.02	-0.02	-0.07	0.05	-0.12	0.00	0.15	0.12	0.04	0.20	0.02	0.22	0.24	0.12	-0.09	-0.17	0.21	0.19	0.11	0.01	-0.06	*