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‘Temporal variation in the sex ratio of a colonial raptor: the case of Eleonora’s falcon (*Falco eleonorae*)’

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Preface

This thesis was conducted under the supervision of Dr. Xirouchakis Stavros at the Natural History Museum of Crete. All of the genetic analyses were carried out under the supervision of Dr. Tsaparis Dimitrios at the Genetics Lab of the Institute of Marine Biology, Biotechnology and Aquaculture at the Hellenic Center of Marine Research.

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Abstract

Eleonora’s falcon (*Falco eleonorae*) is a long-distance migratory bird of prey, which winters in Madagascar and breeds in the Mediterranean Sea, with Greece holding up to 90% of the global population. The species breeds later than any other bird species globally, having synchronized its breeding period with the migration of the small passerine birds, upon which it feeds and raises its young. Clutch size ranges between 1 to 3 eggs with female chicks being generally larger than males, and tend to be more dispersive. Even though there are several studies regarding the biology and behaviour of the species, little is known about their sex ratios. Studying sex ratios of wild animals not only aids the monitoring of reproductive health and growth rates of their populations, but also provides valuable information regarding the evolution of the species. In natural populations, offspring sex ratios can greatly vary from parity (1:1 sex ratio) and the observed biases are generally considered to be species-specific adaptive strategies that are shaped by natural selection. In birds, offspring sex ratio biases are correlated with specific factors, such as season and order of hatching, food abundance, parental quality or social environment. In the only study on the sex ratios in Eleonora’s falcon, a temporal variation in the offspring sex ratio was observed, with females outnumbering males early in the breeding season. The aim of this study was to assess the sex ratio of Eleonora’s falcon offspring by using molecular sexing techniques and to associate any observed variations in the proportion of sexes with specific ecological and biological factors. Additionally, the sexual size dimorphism of offspring was investigated. The results showed a slightly male-biased overall sex ratio (Sex ratio = 0.51) that varied temporally, being female-biased early in the breeding season, as well as early within a brood. The sex ratio also varied with nest orientation and brood size, but did not differ with nest type. Female chicks were shown to be 11% heavier than males, and females were larger than males independently of rank, season, or brood size. The consistency in the results across years, together with the unequal costs of daughters and sons suggests an adaptive sex ratio bias scheme that is being regulated by different sex allocation strategies depending on the parental quality, the abundance of food and the quality of the habitat. However, continued detailed research is necessary to safely interpret the adaptive significance of the observed bias in sex ratio, and to reject the likelihood of artefacts.

Key words: Migratory bird, Aegean Sea, fledglings, molecular sexing, GLMMs, evolutionary adaptation

Περίληψη

Ο Μαυροπετρίτης (*Falco eleonorae*) είναι ένα μεταναστευτικό γεράκι, που διαχειμάζει στη Μαδαγασκάρη και αναπαράγεται στη Μεσόγειο. Η Ελλάδα φιλοξενεί περίπου το 90% του παγκόσμιου πληθυσμού του, το οποίο εντοπίζεται στις ακατοίκητες βραχονησίδες του Αιγαίου. Το είδος είναι μοναδικό στην τάξη των πτηνών διότι φωλιάζει αργότερα από κάθε άλλο είδος παγκοσμίως δηλαδή από τα τέλη Αυγούστου μέχρι τα τέλη Σεπτέμβρη. Την περίοδο αυτή ο Μαυροπετρίτης εκμεταλλεύεται το μεταναστευτικό κύμα των στρουθιομόρφων από την Ευρασία προς την Αφρική για την ανατροφή των νεοσσών του. Αντίθετα, κατά την προ-αναπαραγωγική περίοδο (Μάρτιο-Ιούνιο) το είδος εξαρτάται διατροφικά από ιπτάμενα έντομα που συλλαμβάνει στην πλησιέστερη χερσαία μάζα κοντά στις νησίδες των αποικιών του. Ανάλογα με την διαθεσιμότητα τροφής και την ηλικία/εμπειρία των γεννητόρων το μέγεθος της ωοαπόθεσης (clutch size) κυμαίνεται από 1 έως 3 αβγά. Τα θηλυκά είναι μεγαλύτερα σε μέγεθος από τα αρσενικά, ενώ επιπλέον έχουν την τάση να διασπείρονται περισσότερο. Παρά το πλήθος των μελετών που έχουν πραγματοποιηθεί σχετικά με τη βιολογία και τη συμπεριφορά του είδους, έως σήμερα υπάρχει ένα μεγάλο κενό έρευνας ως προς την αναλογία φύλου που εμφανίζουν οι πληθυσμοί του. Η μελέτη της αναλογίας φύλου αποτελεί ένα ερευνητικό τομέα που όχι μόνο συνεισφέρει στην παρακολούθηση της αναπαραγωγικής υγείας και των ρυθμών ανάπτυξης των άγριων πληθυσμών, αλλά ακόμη μπορεί να προσφέρει σημαντικές πληροφορίες σχετικά με την εξέλιξη των ειδών. Σε πολλά είδη, η αναλογία φύλου κατά τη γέννηση έχει βρεθεί πως διαφέρει από την ισορροπία (αναλογία φύλου 1:1) και αυτή η μεροληψία θεωρείται πως σχετίζεται με ειδο-ειδικές στρατηγικές που διαμορφώνονται από τη φυσική επιλογή. Ειδικότερα στα πτηνά, η μεροληψία που παρατηρείται στην αναλογία φύλου έχει συσχετιστεί με διάφορους παράγοντες όπως είναι η περίοδος και η σειρά εκκόλαψης των νεοσσών, η διαθεσιμότητα τροφής και ο φυλετικός διμορφισμός, η κατάσταση των γονέων αλλά και το κοινωνικό περιβάλλον. Στη μοναδική έρευνα που πραγματοποιήθηκε για τη διερεύνηση της αναλογίας φύλου στο Μαυροπετρίτη παρατηρήθηκε μικρή μεροληψία προς τα αρσενικά, ενώ το ποσοστό των θηλυκών ήταν στατιστικά σημαντικά μεγαλύτερο από εκείνο των αρσενικών στις αρχές της αναπαραγωγικής περιόδου. Λαμβάνοντας υπ' όψιν όλα τα παραπάνω, η συγκεκριμένη εργασία είχε ως σκοπό τη μελέτη της αναλογίας φύλου σε νεοσσούς Μαυροπετρίτη, καθώς επίσης και τη συσχέτιση της τυχόν παρατηρούμενης μεροληψίας με συγκεκριμένους οικολογικούς και περιβαλλοντικούς παράγοντες. Επιπλέον εξετάστηκε η παρουσία φυλετικού διμορφισμού στους νεοσσούς. Η περιοχή μελέτης ήταν μια μικρή αποικία 40 -50 ζευγαριών στη νοτιοανατολική Κρήτη, η οποία διερευνήθηκε σε ένα διάστημα 12 ετών. Τα αποτελέσματα της έρευνας έδειξαν στο σύνολο των ετών μια μικρή μεροληψία προς τα αρσενικά (Αναλογία φύλου = 0.51), η οποία δε βρέθηκε στατιστικά σημαντική. Παρ’ όλα αυτά, η αναλογία φύλου βρέθηκε να μεταβάλλεται μέσα στο χρόνο, με τους θηλυκούς νεοσσούς να γεννιούνται πρώτοι κατά τη διάρκεια της αναπαραγωγικής περιόδου, αλλά και πρώτοι σε μία φωλιά. Επιπλέον, η αναλογία φύλου παρατηρήθηκε να μεταβάλλεται με τον προσανατολισμό της φωλιάς και το μέγεθος της γενιάς (brood size), με τη συχνότητα των θηλυκών νεοσσών να είναι μεγαλύτερη σε ανατολικές και νοτιο-νοτιοδυτικές φωλιές, αλλά και σε μεγάλα μεγέθη γενεών. Ακόμη, διερευνώντας το φυλετικό διμορφισμό στους νεοσσούς, το βάρος των θηλυκών βρέθηκε κατά 11% μεγαλύτερο από εκείνο των αρσενικών, με τη διαφορά αυτή να μην εξαρτάται από την περίοδο και τη σειρά εκκόλαψης ή το μέγεθος της γενιάς. Σύμφωνα με τη βιβλιογραφία, η παρατηρούμενη μεροληψία προς τα θηλυκά μπορεί να οφείλεται στην κατάσταση των γονέων η οποία εξαρτάται από την ηλικία και εμπειρία, αλλά και τη διαθεσιμότητα τροφής. Πιο συγκεκριμένα, γονείς σε καλύτερη κατάσταση θα επενδύσουν στο ‘ακριβότερο’ φύλο, όπου στην περίπτωση του Μαυροπετρίτη είναι τα μεγαλύτερα σε μέγεθος θηλυκά. Επιπλέον, η επαναληψιμότητα των αποτελεσμάτων με την πάροδο των χρόνων, αλλά και η συμφωνία των αποτελεσμάτων με προηγούμενη αντίστοιχη έρευνα στο Μαυροπετρίτη υποδεικνύουν πως η παρατηρούμενη μεροληψία μπορεί να οφείλεται σε ειδο-ειδική προσαρμογή η οποία βασίζεται σε συγκεκριμένη στρατηγική του είδους που σχετίζεται με την κατάσταση των γονέων, τη διαθεσιμότητα της τροφής και την καταλληλότητα του ενδιαιτήματος. Ωστόσο, απαιτείται συνεχής λεπτομερής έρευνα για την ασφαλή ερμηνεία της προσαρμοστικής σημασίας της παρατηρούμενης μεροληψίας στην αναλογία του φύλου, καθώς και για την αποφυγή τυχόν τεχνικών σφαλμάτων (artefacts).

Λέξεις κλειδιά: Μεταναστευτικό πτηνό, Αιγαίο Πέλαγος, νεοσσοί, μοριακός προσδιορισμός φύλου, GLMMs, εξελικτική προσαρμογή

1. Introduction

1.1. The study organism Eleonora's falcon (*Falco eleonorae*)

Species description

Eleonora's falcon (*Falco eleonorae*) is a medium-sized bird of prey of the Falconidae family, which together with its close relatives, *Falco concolor* and *Falco subbuteo*, belong to the Hypotriorchis subgenus (Wink *et al.*, 1998). As many other raptor species, Eleonora's falcon exhibits reversed sexual size dimorphism with females to be larger than males, with an average weight of 399 ± 27 g for females and 327 ± 22 g for males (Ristow and Wink, 2000; Wheeler and Greenwood, 1983). At the same time, there is a colouration pattern of the ceres and the eye – rings that varies between sexes during the breeding season; ceres and eye - rings of adult females are bluish-green, whereas those of adult males are orange – yellow (Ristow and Wink, 2000) (Figure 1.1.1.). In addition, the species is polymorphic and adults are characterized by a light and a dark morph, although an intermediate plumage variation has been described as well (Ristow and Wink, 2000).

Figure 1.1.1. Adult male (left) versus adult female (right) Eleonora's falcon. The cere and eye – ring of the male are yellow, while in the female are blue (iNaturalist, 2021; N.H.M.C.).

Distribution and habitat

Eleonora’s falcon is a long – distance migratory bird, with a very wide range. It can be found from its breeding grounds in the Mediterranean Sea and the eastern Atlantic, to Madagascar and the surrounding islands, where it overwinters (López *et al.*, 2009) (Figure 1.1.2.). In Greece, the species is largely distributed in the Aegean Sea, from Crete to NE Aegean, with most of the breeding colonies to be located in Dodecanese (n = 83) and Cyclades (n = 73), according to the last national census (Dimalexis *et al.*, 2008).

Figure 1.1.2. Eleonora’s falcon distribution. The breeding colonies are in red, the wintering habitats are shown in blue (Orta *et al.*, 2020).

On its breeding sites, Eleonora’s falcon lives in colonies located mainly in remote, uninhabited islets or inaccessible and steep, coastal cliffs on larger islands (Dimalexis *et al.*, 2019). The nests can be found in cliff ledges, caves, holes, slopes, under vegetation cover or even on flat ground (Vaughan, 1960) (Figure 1.1.3.). The distance between nests depends on the colony characteristics and can vary from 2 m to 20 – 50 m (Hadjikyriakou *et al.*, 2020). These peculiar landscapes and nest sites provide a safe environment for breeding, attenuating the human presence or the predation of other terrestrial hunters, while the cliff heights act as a training terrain for the first flights of the young individuals (H.O.S., 2021).

Figure 1.1.3. A typical habitat for Eleonora’s falcon; isolated, steep coastal cliffs (iNaturalist, 2021).

Population and conservation status

The global population of Eleonora’s falcon is estimated to be 29,200-29,600 mature individuals (BirdLife International, 2021). Greece holds up to 90% of the global breeding population, with over 12,000 pairs breeding in the Aegean. At a global level, the species is classified as Least Concern (BirdLife International, 2021). Nevertheless, it is included in Annex I of the EU Directive 2009/147/EC on the conservation of wild birds, as well as in Annex II of the Bern Convention and categorized as ‘SPEC 2’ according to BirdLife International, which means that is a ‘species with an unfavourable European conservation status, and with more than half of the global breeding or wintering population concentrated in Europe’, thus requiring special conservation measures (BirdLife International, 2021; European Parliament, 2009; Bern Convention, 1979).

Migration and dispersal

Migration comprises one of the most crucial stages of the biological cycle of Eleonora’s falcon (López, *et al*., 2009). The falcons are able to travel more than 9,000 km in a straight line to reach their breeding grounds in the Mediterranean and the eastern Atlantic, from Cyprus to the Canary Islands (Dimalexis *et al.*, 2019; Gschweng *et al.*, 2008). This migration route occurs every spring between March and May. However, immature and non – breeding individuals can, also, be found inland, in Africa, the Middle East and continental Europe (Wink, 1995; Walter, 1979). When the breeding season is over, between October and December, all the individuals migrate back to their wintering habitats in Madagascar and the Mascarene islands. During this autumn migration, it has been observed that juveniles migrate independently of the adult falcons, a behaviour that implies the inheritance of the navigating information (Gschweng *et al*., 2008).

Eleonora’s falcon individuals develop strong bonds with their breeding and natal grounds, as well as with their partners. Adults exhibit great site tenacity by returning to the same breeding colony every year, while young individuals tend to return for nesting close or at their natal colonies, thus manifesting a high degree of philopatry (Ristow and Wink, 1979; Ristow and Bourdakis, 1999*)*. In spite of that, males are more likely to be philopatric, while females are more prone to dispersal and can easily migrate to other colonies (Ristow and Wink, 2000).

Breeding biology

The species is characterized as monogamous, without exhibiting extra – pair copulations (Ristow and Wink, 2000). Even though individuals return to their breeding colonies in April, to occupy the most suitable territories and secure their partners, courtship begins in July (Ristow and Wink, 2000; Ristow and Bourdakis, 1999). Egg - laying starts in the second half of July and by the beginning of August the majority of eggs is laid. One to three eggs – rarely four eggs – are laid in each nest, with the first two chicks hatching within 36 hours and the third chick hatching 2 to 3 days later (Ristow and Wink, 2000). Generally, 3 – egg clutches tend to be laid earlier in the breeding season and within a clutch the third egg is smaller and darker (Wink, 1985). The incubation of the eggs lasts approximately 28 - 33 days, indicating that by the beginning of September most of the nestlings hatch and chicks are fully fledged and ready to fly 40 days later (Ristow and Wink, 2000; Dimalexis *et al.*, 2019). By the first week of

November, most of the colonies are deserted (Ristow and Bourdakis, 1999). Males will start breeding at the age of 3 to 4, whereas females tend to breed earlier at the age of 2 (Ristow and Wink, 2000)..

Feeding ecology

Eleonora’s falcon represents the latest breeder of all the summer visitors in Europe (Dimalexis *et al.,* 2008). This unique characteristic derives from its peculiar foraging behaviour, which includes feeding with insects throughout the year, except for the post – hatching season when Eleonora's falcon individuals switch their diet to small passerine birds that migrate back to their southern wintering grounds (Xirouchakis *et al.,* 2019; Ristow and Wink, 2000). Especially in the pre – breeding period, falcons may fly hundred kilometers away from their nests in search for insect – rich habitats, such as forested or cultivated areas, rivers and lakes both in mountainous or low altitude areas, in the mainland, as well as on large islands in the Aegean (Dimalexis *et al.*, 2019). The insect prey consists mainly of the Coleoptera, Homoptera, Orthoptera and Hymenoptera taxa. In contrast, breeders use to hunt in short trips close to the colony, feeding on a variety of birds (approximately 120 species) with warblers, shrikes and thrushes to be the most dominant ones (Xirouchakis *et al.,* 2019). During days with unfavourable weather conditions, individuals may also feed with insects in the nearby mainland to supplement their diet (Dimalexis *et al.,* 2019; Xirouchakis *et al.,* 2019). The mean flight altitude that has been observed is 1292 masl, with the maximum to reach 3500 masl (Xirouchakis and Panuccio, 2019). Wind and time play a crucial role in Eleonora’s falcon foraging activity. During windy days their most common flying technique is hovering against the wind, creating a flying trap for the passing prey. They usually hunt intensively during sunset and their flight altitudes vary according to the daily temporal passerine migration patterns (Xirouchakis and Panuccio, 2019).

Threats and limiting factors

Although Eleonora’s falcon population status has been described as increasing (BirdLife International, 2021), the species’ unique life history - such as their strong bonds with their natal colonies and even partners - in combination with a consistently changing environment, make Eleonora’s falcon vulnerable to various potential threats (Ristow and Wink, 1989).

Climate change is one of the most significant factors that might negatively impact the species (Dimalexis *et al.,* 2019). Mediterranean ecosystems have been considered among the most likely impacted by climate change, mainly due to precipitation decrease, increase of temperature, drought and wildfires. To this extent, breeding birds in the Mediterranean is predicted to shift their breeding habitats more than 500km northeast by the end of 21st century (Dimalexis *et al.*, 2019). Hence, Eleonora's falcon is expected to lose the largest part of its current breeding range, while given the species' specialization and preference in habitat and diet composition, this relocation might result in decline of population size, instead of colonization of new habitats (Dimalexis *et al.*,, 2019). At the same time, climate change is expected to shift the migration period of passerines, leading to the deprivation of synchronization between nestlings' hatching and the migratory passage that is responsible for the species' breeding success (Dimalexis *et al.,* 2019).

Another major threat to which Eleonora's falcon is currently exposed and will be in the future is the human disturbance (Ristow and Bourdakis, 1999). Touristic development or infrastructure development (e.g. wind turbines) at or near colonies that will result in intense noise or even habitat exploitation, will cause birds to leave their eggs unprotected, exposed to the sun or predators, thus impacting species' breeding success (Ristow and Bourdakis, 1999). Lastly, anthropogenic intervention, such as the intense use of pesticides in agricultural areas that constitute important feeding grounds for the pre – breeding period of Eleonora's falcon might reduce its food basis (Ristow and Bourdakis, 1999).

1.2. Sex ratios in the wild

Generally, sex ratio refers to the ratio of males to females in a population (Sapir *et al.,* 2008). However, in a variety of studies, sex ratio is defined as the proportion of males or females in a population. These definitions can be categorized, according to the point of time they are referring to, resulting in three types of sex ratio: i) 'primary sex ratio': the sex ratio at the time of fertilization (egg-stage), ii) 'secondary sex ratio': the sex ratio at birth or hatching (nest stage) and iii) 'tertiary sex ratio': the sex ratio after fledging (adult stage) (Mayr 1939).

Sex ratio is a parameter that not only aids monitoring of reproductive health and growth rates of a population, but is also a very useful trait for answering general evolutionary questions (Terrell *et al.,* 2017; Hardy and Boulton, 2019). Especially, when sex ratio is described in relation to the specific life – history characteristics that a species possesses, it is vital in understanding the past, present and future of a population (Skalski *et al.,* 2005). As a result, sex ratios are the focal subject for both ecological and evolutionary research (Sapir *et al.,* 2008).

Fundamentals of sex ratio theory

In the wild, it is widely observed that vertebrates have the ability to alter the amount of energy that goes into male versus female reproductive output at a variety of developmental levels, depending on the environmental and social conditions that surround them (Navara, 2018). Thereby, in natural populations the proportions of females and males can greatly vary, often resulting in sex ratios that deviate from parity (1:1 ratio). The theory that explains populations' sex ratios, by combining evolutionary and behavioural approaches, together with mathematical models and empirical testing is called **sex ratio** or **sex allocation theory** (Hardy and Boulton, 2019). Sex ratio theory is, basically, a broad concept that provides a framework for understanding a range of reproductive behavioural patterns, with an emphasis on the sex ratios of the progeny that parents produce (Hardy and Boulton, 2019).

For many years, in species with genetic sex determination, such as birds and mammals, sex determination was assumed to be random, without being controlled by the parents. However, over the last 50 years more and more scientific publications have proven that a huge variety of animals are able to manipulate their offspring sex in order to increase their fitness and this behaviour could be shaped by natural selection (Davies *et al*., 2012). To further understand the several patterns of sex allocation, the major ideas regarding the distribution of sex ratios in natural populations are briefly described bellow.

Fisher's equal investment hypothesis

Fisher's theory is a null model of sex allocation developed by Ronald Fisher (1930), although the conception of the idea dates back to Darwin (Edwards, 1960). Fisher postulated that sex ratios can be shaped by natural selection, and all else being equal, a 1:1 ratio is favoured. In other words, frequency – dependent selection should lead to the equal production of females and males in a population. Frequency - dependent selection occurs when the fitness of a genotype or phenotype in a population is affected by its frequency in the population (Ayala and Campbell, 1974) (Figure 1.2.1.). However, in cases when energy costs for raising sons and daughters are different, natural selection will not favour a 1:1 sex ratio, but an equal investment between the sexes, even if this would result in the overproduction of one sex. Considering that the individuals try to maximize their fitness i.e. the passing on of their genes to the next generations with the least cost, selection would assure this by favouring equal parental expenditure on the sexes (Davies *et al.*, 2012).

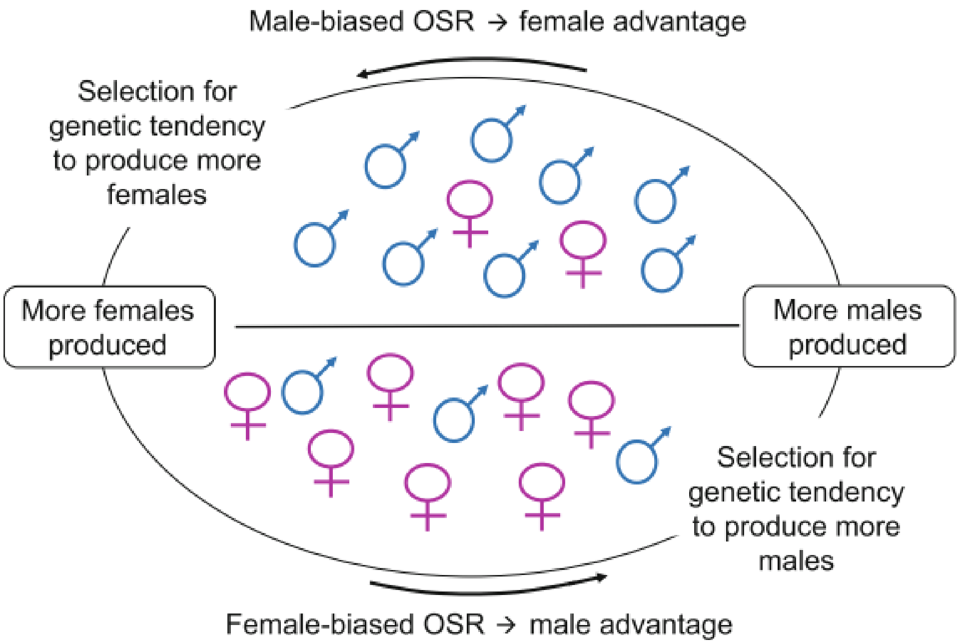


Figure 1.2.1. Fisher's theory of equal investment as explained by Hamilton (1967). Operational sex ratio (OSR) is the ratio of sexually competing males that are ready to mate to sexually competing females that are ready to mate. When OSR is female biased, selection will favour the individuals with a genetic tendency to produce more male offspring. This will result in a 1:1 ratio of males to females, until OSR becomes male biased and the reverse selection process occurs (Navara, 2018).

Sex allocation when relatives interact

Fisher assumed that there were no interactions between relatives, either cooperatively or competitively (Davies *et al.*, 2012). Conversely, Hamilton (1967) pointed out that the sex which competes its siblings the most, is less efficient in passing on its genes to the next generations (Komdeur and Pen, 2002). If, for example, in a population daughters compete for resources, a male – biased sex ratio is favoured by selection to reduce competition. This is called Local Resource Competition (Clark, 1978). A special type of Local Resource Competition is the Local Mate Competition as presented by Hamilton (1967). The theory suggests that if siblings compete each other for mates, the sex ratio should be female biased. Research on Local Mate Competition was mainly focused on insects such as wasps and ants, as those animals experience strong attachment with their natal groups, thus high competition for mating (Griffiths and Godfray, 1988). Alternatively, in cases when relatives cooperate instead of competing, offspring of one sex are more likely to remain close to their group, in order to assist further in the rearing of the offspring and thus, sex ratio should be favoured towards the helping sex (Davies, *et al.,* 2012). This is called Local Resource Enhancement.

Maternal condition hypothesis

The third major idea of sex allocation was described by Trivers & Willard (1973). They proposed that sex ratios of females to males can be skewed in response to specific parental conditions. They research focused on a population of a polygamous species, where males were competing for access to females and in which three assumptions held: i) mother in better condition had more resources for reproduction and thus, produced offspring of better quality; ii) higher quality offspring would develop in higher quality adults; and iii) a healthy adult son would be more beneficial than a healthy adult daughter, as stronger males would be more capable of competing other males for mating. Therefore, Trivers and Willard concluded that in this polygynous system where males use costly ornaments to defend their territories, producing a male that is of high quality to compete other males for mating might be energetically more costly. However, when resources are abundant and mothers are able to pass down their good condition to their offspring, the benefit of producing males will offset the cost (Trivers and Willard 1973; Davies *et al.,* 2012).

1.3. Sex ratios in birds

Drivers of sex ratio variation

In nature, a variety of examples of biased avian sex ratios have been observed. To date, particularly regarding offspring sex ratios, there have been at least 130 studies that indicate skewed sex ratios at birth (Navara, 2018). All of these studies point out that the variations in birds' sex ratios are associated with specific factors that can affect the allocation of the sexes within a population. The most well - studied drivers of offspring sex ratio variations, as summarized by the results of several studies, are: *season of hatching, hatching order, food availability and sexual size dimorphism, parental quality, social environment and clutch size*.

Season of hatching

In many bird species the time of breeding has been found to affect the sex ratio of the offspring. Daan *et al.,* 1996 examined this relationship in diurnal raptors where he found different season – related sex ratio patterns among species. In some of the species, such as goshawk, sparrowhawk and marsh harrier, the sex ratio was male – biased the later the clutch was laid. However, in the falcon species he examined, such as European kestrel and lesser kestrel, sex ratios were female biased while laying date increased (Sheldon, 1998). He explained those patterns by taking into account the different life histories of the two groups of birds. In the two falcon species males breed earlier than females and a male's probability of successful breeding increases the earlier it is born, something that does not apply to females. The opposite pattern exists in the three larger species (Sheldon, 1998). Thus, an early hatching might imply a successful reproduction for the sex whose maturation occurs earlier.

Another group of studies suggested that food abundance might affect the seasonal variation in the sex ratios. In most species of birds that exhibit sexual size dimorphism, the sex ratio was seasonally biased with a greater production of the most expensive sex, when food was more abundant (Dijkstra *et al.*,1990; Weatherhead, 1983). Thus, as food abundance declines across the breeding season, the reproductive value of the larger and consequently more 'costly' sex is expected to decrease, since insufficient resources will not support a larger chick (Sheldon, 1998).

Hatching order

A common behaviour in birds is the reduction of the brood, which most of the times occur due to the increased mortality of the later – hatching nestlings and the enhanced survival of the earlier - hatching ones within a brood (Magrath, 1989). In harsh environmental conditions, such as food shortage, the nestlings of the larger sex whose rearing demands a lot of parental investment, are in greater risk of mortality, especially when they hatch from the last egg within a clutch (Torres and Drummond, 1997). For this reason, as the proportion of food that parents provide to their nestlings usually decreases with hatching order, the death of the nestling that hatched last is more probable if it belongs to the costlier ('expensive') sex and especially when the brood reduction is significantly likely to occur (Benito and González-Solís, 2007). Thus, to prevent or reduce this unfavourable brood reduction, which potentially may reduce parent fitness, the costlier sex is expected to be produced in higher frequencies earlier in the hatching order ('Intra – brood sharing – out hypothesis') (Carranza, 2004). A clear pattern of skewed first - rank sex ratios was observed in bald eagles (*Haliaeetus leucocephalus*), where sexes within broods were allocated in such a way (i.e. higher percentage of first hatched females) that mortality due to siblicide was minimized, and hence parents could obtain the maximum of their fitness (Bortolotti, 1986b).

In contrast, the ‘intra-brood competitive equilibrium’ hypothesis suggests that when an offspring of the smaller sex might be in disadvantage due to increasing competition with its siblings of the larger sex and mainly when hatches last within a clutch, the adaptive strategy for the parents would be the larger sex to hatch last in the hatching sequence, in order to avoid the probability of starving to death (Uller, 2006).

Food availability and sexual size dimorphism

If the sexes of offspring differ in size, competition between nestling sexes might arise due to the relative size differences and the higher energetic requirements of the larger sex (Daan *et al.,* 1996; Fiala and Congdon, 1983). In this way, the larger sex is more exposed to disadvantageous conditions such as food scarcity (‘Costly sex hypothesis’). Several studies have successfully attempted to find a connection between the ‘Costly sex hypothesis’ and sex ratios. In tawny owls (*Strix aluco*) it was found that primary sex ratio within broods was biased towards the larger sex in prey - abundant territories (Sheldon, 1998). Similarly, supplementary feeding on kakapos (*Strigops habroptilus*) mothers before breeding, resulted in a extreme bias towards the larger sex (Clout *et al.,* 2002). In general, the smaller sex is preferred under adverse environmental conditions and lower food supplies, whereas the larger sex is preferred under benign conditions (Benito and Gonzalez-Solis, 2007).

Parental quality

It was postulated by Trivers & Willard (1973) that the environment and condition that mothers provide for their offspring can impact their reproductive success and thus, inducing sex – specific selection. Even though this suggestion first implied that the mother might transmit their physical condition to the offspring through parental care, it is generally considered that any quality of the parents can be transmitted to the offspring (Sheldon, 1998). The condition of a bird might be related, for example, to the quality of its territory, its ability to obtain food successfully (Wiebe and Bortolotti, 2004) or gaining access to the safest nests (Jaatinen *et al.,* 2013), or to a secondary sexual trait of the parent that might give a higher reproductive value to the sex that possess it (Sheldon, 1998). In this way, parents would favour the sex that will profit the most by their good condition even if this sex might cost a lot to produce, consequently achieving the maximum fitness (Trivers and Willard, 1973).

For instance, in collared flycatchers (*Ficedula albicollis*) when males rearing a brood had a large forehead patch, the brood sex ratio was male biased. The large forehead patch was found to be a heritable trait, which gave a reproductive advantage to the males that had it (Ellegren *et al.,* 1996;

Sheldon, 1998). In another experiment in lesser black-backed gulls (*Larus fuscus*), mothers were, first, objected to removal of their eggs while they were laid, in an attempt to decrease their condition since they had to compensate for the lost eggs by producing more and, second, some of them received supplementary food afterwards. The results showed that mothers supplemented with food (i.e. in good condition) managed to maintain a 1:1 sex ratio in their clutches and produced larger eggs compared to the unsupplemented ones which significantly produced less of the larger sex (Nager *et al.,* 1998; Navara, 2018).

Social environment

The social environment, such as competition or cooperation between siblings has been found to favour unequally sexes in birds (Navara, 2018). In a proportion of birds, young return to their natal colonies in order to help with the rearing of their future siblings. This phenomenon is mainly observed in males that are philopatric, whereas in general female birds are dispersive. Thus, since the philopatric sex might be objected to Local Resource Competition when habitat quality is low, parents bias the sex of their offspring towards the dispersive one namely females (Sheldon, 1998). For example, in the Seychelles warbler (*Acrocephalus sechellensis*), where the female is the philopatric sex, birds breeding on high quality territories produce significantly skewed sex ratios towards females, whereas in low quality territories the sex ratio is biased towards males who disperse (Komdeur *et al*., 1997). Paralleling the effect of social environment and parental quality on the variation of sex ratios, parents in good condition that are above average of occupying nest sites or food resources should invest in the philopatric sex with the condition that the paternal phenotypic quality is inherited by the offspring (Leimar, 1996).

Clutch size

An alternative theory to Trivers & Willard suggests that when environmental conditions are poor, parent birds overproduce the ‘cheapest’ sex or alternatively the sex that is less sensitive to harsh environmental conditions (Myers, 1978). In contrast, in White-throated Dippers (*Cinclus cinclus*) where males are larger than females, thus sons are more expensive to raise than daughters, the largest clutches were female-biased suggesting that skewing the sex ratio towards the ‘cheapest’ sex is important when the number of chicks in a brood is high and parents should provide sufficient parental care to all of their offspring (Øigarden and Lifjeld, 2013).

Table 1.1. Summary of the drivers of sex ratio variation in birds

Factor	Expected offspring sex ratio bias	
1. Season of hatching	**If maturation time differs between sexes:** *early in season → more of the larger sex*	**If there is sexual size dimorphism:** *early in season → more of the larger sex*
2. Hatching order	**If brood reduction will lead to the mortality of the late-laid nestlings:** *early in hatching order → more of the 'costlier' sex* *('Intra – brood sharing – out hypothesis')*	**If there is increased competition within brood due to sexual size dimorphism:** *early in hatching order → more of the smaller sex* *('Intra-brood competitive equilibrium hypothesis')*
3. Food abundance & size dimorphism	**If there is sexual size dimorphism:** *benign conditions & food abundance → bias towards the larger sex*	
4. Parental quality	**If the progenitor value is the main driver of the sex ratio bias** *parents in good condition → bias towards the 'costlier' sex or the sex from which their fitness might profit the most (Trivers & Willard, 1973)*	
5. Social environment	**If there is sex – specific philopatric or dispersive behaviour and competition for local resources:** *high – quality habitat & parents in good condition → bias towards the philopatric sex ('Local resource competition/enhancement hypothesis')*	
6. Clutch size	**If the environmental condition is the main driver of the sex ratio bias:** *harsh environmental conditions → overproduction of the 'cheapest' sex or the less – sensitive sex*	**If the sufficient parental care and survival of the whole clutch is the main driver of the sex ratio bias:** *large clutches → overproduction of the 'cheapest' sex*

Potential mechanisms of sex manipulation in birds

Although variations in the offspring sex ratios of wild birds, either on the population or family level, have widely been observed, the question that still remains unclear is how exactly wild birds can bias the sex ratios of their offspring. In birds, females are the heterogametic sex by demonstrating a ZW system of genetic sex determination, indicating that females determine the sexes of their offspring by donating either a Z or W chromosome to an egg (Komdeur and Pen, 2002). This means that after meiosis, each oocyte in the ovary of a female consists of both Z and W chromosomes and hypothetically can ultimately produce either a male or female offspring. In general, sex manipulation it is suggested to occur during two developmental periods: before egg - laying and after egg - laying.

Before egg - laying, after the phase of meiosis I the oocyte contains one pair of homologous chromosomes (either W or Z), whereas the other pair is expelled into the unfertilizable polar body (Navara, 2018). During the phase of meiosis I, it is believed that oocytes might be predestined to maintain a particular sex chromosome, and then females might have a kind of mechanism to select which occytes and with which hierarchy will ovulate and consequently hatch at the optimal time (Badyaev *et al*., 2006). In addition, the time and the duration of the yolk formation in an oocyte might affect the sex of an egg. This can be explained by the fact that environmental changes, such as stress or tension by territorial intrusion are able to alter a variety of hormones, or immunological or physiological factors that females deposit into the yolks of their eggs (Navara, 2018). For example, eggs that produced more males had accumulated larger quantities of specific hormones but follicles that grew faster produced more females (Badyaev *et al*. 2006; Young and Badyaev, 2004). Lastly, epigenetic regulation might result in binding of specific proteins to the chromosomes, thus altering their potential in the meiotic division (Navara, 2018).

After meiosis, sex – specific follicle atresia might occur. Atretic follicles fail to ovulate, resulting in reabsorption of the yolk and disintegration of the follicle. The atretic follicles have been induced in chickens via fasting or corticosterone treatment (Johnson, 2015). Also, blocking of oocytes carrying a specific chromosome by female’s inhibitor proteins or by selective binding of the sperm might potential manipulate the sex of the egg.

Additionally, hormones can potentially influence the sex of an egg. It is known that food shortage or a low maternal body condition can trigger the response of mediators that indirectly interact with the endocrine system or directly impact the reproductive system by stimulating or inhibiting the function of the reproductive organs. For instance, in birds treatments with hormones prior to the completion of meiosis could effectively manipulate which sex chromosome the egg contains at ovulation (Navara, 2018).

On the other hand, sex manipulation might occur after the laying of the eggs via sex – specific mortality and sex biased provisioning. In many cases, parents selectively reject their chicks or preferentially feed more the same sex a behaviour that can lead to sex – specific mortality between offspring (Clotfelter, 1996). Lastly, sex – specific mortality might be the effect of some maternal hormones (Komdeur and Pen, 2002).

Sex ratio variation in Eleonora's falcon

So far, only one study has investigated the variation of the sex ratios in Eleonora's falcon. The study was conducted in a small colony of the Aegean during five consecutive years (Ristow and Wink, 2004). The results showed a seasonal variation in Eleonora's falcon offspring sex ratios, with daughters to be significantly more abundant during the beginning of the hatching period and sons to dominate in the middle and final period. Overall, the sex ratio was slightly male biased. In addition, three – egg clutches were found to be more frequent early in the season and the first egg laid was the heaviest within a clutch. The authors proposed that the early bias towards females indicated a higher degree of fitness of the parents, particularly of males, since in a previous study in Eleonora's falcon larger males were found to have larger clutches (Wink *et al.*, 1985). Overall, according to this study successful and experienced Eleonora's falcons, tended to start breeding earlier and this resulted in an early bias towards female offspring. However, a major limitation of this study was the inaccessibility of most of the nests located on steep cliffs, which according to the theory comprise the most suitable territories for the rearing of the young and consequently might lead to missing information regarding the sex ratio skews found in this colony.

Aim and objectives

The data of this study were collected for a long – term monitoring project of the Natural History Museum of Crete, based on the Eleonora's falcon population in the Aegean Sea. The focal area was a small colony in south Crete, consisting of 40 to 50 nests with almost all of them being accessible to researchers. This characteristic feature of the colony, including the life history and knowledge gap on sex ratios of Eleonora's falcon, was a major incentive for studying the following issues:

a) Determination of sex by applying molecular sexing methods
b) Investigation of offspring sex ratios and their temporal variation
c) Association of offspring sex ratios with specific ecological and biological parameters
d) Investigation of sexual size dimorphism, and its association with specific ecological and biological parameters

2. Materials and methods

2.1. Study area

This study was conducted on the Anavatis islet (35°1.839'N, 26°14.132'E) in Xerokampos, southeastern Crete (Figure 2.1.). Anavatis is a rock with a surface of just 1.4 ha (0.01 km ^{2}), a perimeter of 620 m and a maximum altitude of 12 m. It is, also, part of a group of three uninhabited islets called Kavalloi. Approximately 400 m away from the mainland, these steep, small islands are dominated by limestone cliffs and are partially vegetated with halophilous plants, garrigues and Mediterranean shrubs (i.e., Euphorbia dendroides) (Portolou *et al,* 2009; Xirouchakis and Panuccio, 2019).

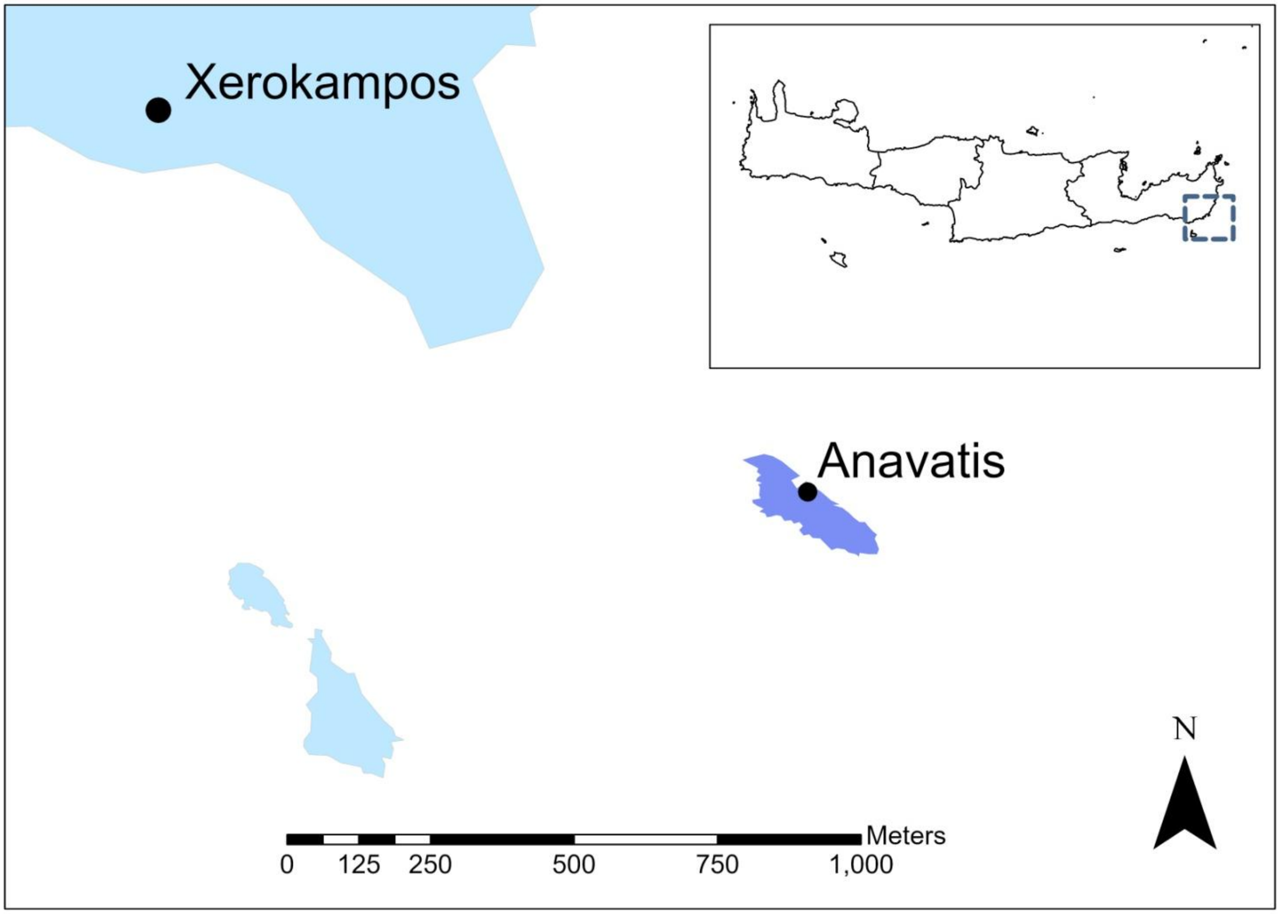


Figure 2.1.1. Study area in southeastern Crete, Greece

This small island provides a suitable breeding habitat for Eleonora's falcon (*Falco eleonorae*), hosting a colony of approximately 100 – 140 individuals or 40 – 50 pairs per year. The area is characterized by a

Mediterranean to subtropical climate, with an extended dry period, between April and mid – November. The mean annual temperature is 20.8°C and the total annual precipitation 300mm (Stavros M. Xirouchakis and Panuccio, 2019).

The study area is part of the Natura 2000 network, listed as an Area of Special Protection (Special Protection Areas, SPA) with a site code GR4320015. In addition, the study site meets the B1iii, B2, C2 criteria, thus being characterized as an Important Bird Area (Important Birds Areas, IBA) according to the Bird Life International project (Table 2.1.1.) (Portolou *et al.*, 2009).

Table 2.1. 1. The table describes further the IBA (Important Birds Area) Criteria that the study area meets

IBA Criteria – *Study area*
B. European • **B1. Congregations:** iii) The site is known or thought to hold ≥1% of a flyway or other distinct population of other congregatory species. • **B2. Species with an unfavourable conservation status in Europe:** The site is one of the ‘n’ most important in the country for a species with an unfavourable conservation status in Europe (SPEC 2, 3) and for which the site-protection approach is thought to be appropriate
C. European Union • **C2. Concentrations of a species threatened at the European Union level:** The site is known to regularly hold at least 1% of a flyway population or of the EU population of a species threatened at the EU level (listed on Annex I and referred to in Article 4.1 of the EC Birds Directive

2.2. Data collection

Field work was carried out between 2009 and 2020, accounting for a study period of twelve years. Field visits started a few weeks after the arrival of the birds in mid – May and lasted until the end of the breeding season, before birds departure for their wintering grounds in October. During each sampling year, nests were visited three times: i) the first visit occurred in May, when all nests and eggs were identified and counted, with each nest and egg receiving a unique number and ID respectively, ii) the second visit occurred in early September, when all young chicks (nestlings) were counted, and finally iii) the third visit occurred in mid – September during which all fledged chicks (fledglings) were counted and ringed. During the third visit on the field, specific measures were taken from the fledglings, such as a) body mass (gr) and wing length (mm). In cases where measures for body mass couldn’t be taken, body mass was indirectly calculated by linear regression of body mass on wing. At the same time, blood (100 ul) or feather samples were collected from each fledgling and stored in Ethanol. All samples were frozen at -20^{o}C until the DNA extraction.

2.3. Molecular sexing

The process of sex identification in sexually dimorphic birds is generally a simple task. However, distinguishing sex in monomorphic birds or young chicks can be challenging (Morinha, Cabral, and Bastos 2012). Most conventional methodologies for sex determination in birds include behavioral observations, measures of morphometric traits, laparoscopy or cytogenic analysis. Nevertheless, most of these techniques tend to be not only expensive, but also time consuming and at some cases quite harmful for the birds (Morinha *et al*., 2012). As a result, molecular sexing techniques have been improved and widely applied, providing an efficient and often less expensive method for sex identification in birds.

The DNA – based sexing techniques involve chromosome specific markers, taking advantage of the variations among the heterogametic sex (females comprise of Z and W chromosomes) and the homogametic sex (males comprise of two Z chromosomes). In birds, this marker is the CHD1 gene (Chromodomain Helicase DNA Binding 1 gene) which encodes an ATP – dependent protein that plays a crucial role in the arrangement of chromatine and the regulation of transcription (Morinha *et al.,* 2012; UniProt 2021). Although the CHD1 gene can be found both in W and Z chromosome in birds, it carries at least two introns that vary in length between the Z and W chromosome (Dubiec and Zagalska-Neubauer, 2006). As a result, the intron – related size differences in the CHD1 gene between the two sex chromosomes may reflect the number of sex chromosomes an individual might possess, and consequently its gender. For this reason, multiple intron – specific primers have been designed and used for sex identification by polymerase chain reaction (PCR) – methods (Griffiths *et al.,* 1996; Kahn *et al.,* 1998).

Eleonora’s falcon individuals have a slight sexual size dimorphism that especially in the developmental stage of the fledgling, impedes the sex determination by morphological traits. Thus, a PCR - based methodology was implemented to identify the sex of fledglings (Ristow *et al*., 2004).

Genomic DNA extraction from blood and feathers

With the exception of mammals, most vertebrates consist of red blood cells that retain a nucleus (Hartenstein, 2006). This characteristic allows for sufficiently high quality and quantity DNA extraction from avian blood samples. For the extraction of genomic DNA from avian whole blood samples, Miller *et al*., 1988 protocol was implemented, with slight modifications (Appendix 1).

Contrarily, feather samples contain much lower amount of DNA than blood or tissue samples (Harvey *et al*. 2006). Thus, in order to obtain high quality and quantity DNA from feathers, the DNeasy® Blood & Tissue Kit from QIAGEN was implemented (Qiagen, 2006).

Following the extraction of the DNA, all total DNA samples were analyzed with 1.5% agarose gel electrophoresis, in order to confirm that DNA was successfully obtained. Agarose was diluted in 1x TAE Buffer (50x: 242g Tris Base, 57.1mL Glacial Acetic Acid, 100mL 0.5M EDTA (pH 8.0), dH_2O) and DNA was stained with Ethidium Bromide. Electrophoresis was performed at a constant voltage of 90 V for 50’ and DNA bands were visualized using a UV light device.

PCR Amplification

A modified version of Kahn *et al*., 1998 PCR protocol was applied to the samples. The primers used for PCR amplification were: a) the forward primer 1237L: 5´-GAGAAACTGTGCAAAACAG-3´ and b) the reverse primer 1272H: 5´-TCCAGAATATCTTCTGCTCC-3´. Prior to PCR, the concentration of all DNA samples was measured in Nanodrop Spectrophotometer and the samples were diluted according to the final concentration of DNA in the PCR mix. PCR mix consisted of: 30 ng (1μL) total DNA in 10uL total volume, 0.3 Primer 1237L (10μM) and 0,3 μL Primer 1272H (10μM), 0.2 μL nucleotide mix (10mM), 1μL Buffer 10x, 0.6 μL $MgCl_2$ (25mM) and 0.04 μL Taq – Polymerase (0.04 Units; KAPA Biosystems). Each PCR reaction started with an initial denaturation at 94^oC for 2 min and amplified for 31 cycles with the following thermal profile: denaturation at 94^oC for 30 sec, annealing at 56 ^oC for 60 sec and extension at 72^oC for 2 min. A 2 min extension at 72 ^oC followed the last thermal cycle. The reaction temperature then lowered to 4 ^oC for further storage.

PCR product electrophoresis in agarose gel

PCR products were separated with electrophoresis using a 3% agarose gel. For the gel preparation, agarose was diluted in 1x TBE Buffer (10x: 108g Tris Base, 55gr Boric Acid, 40mL 0.5M EDTA (pH 8.0), dH_2O) and DNA was stained with MIDORI^{Green} Xtra. Electrophoresis was performed at a constant voltage of 90 V for 2h. DNA bands were visualized and images were acquired using a Green LED transilluminator (Figure 2.2.). The double bands represented a female individual with two different in size copies of the CHD1 gene, whereas the single band a male with two equal in size CHD1 copies .

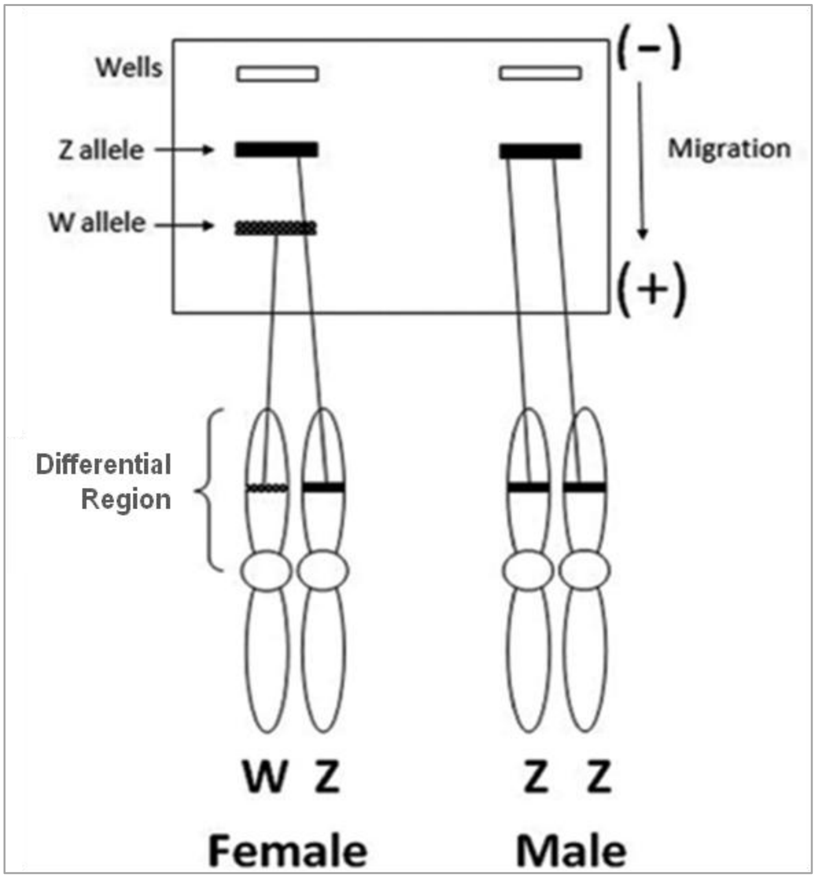


Figure 2.3.1. Illustration of sex identification methodology by using PCR and agarose gel electrophoresis to detect the differences in CHD1 – asscociated intron sizes (Purwaningrum *et al*. 2019)

Genotyping

To verify the sex results obtained by PCR, a proportion of samples were genotyped for the CHD1 gene by using the 1237L forward primer. This technique requires PCR amplification with a labeled primer and then the visualization of the labeled fragments by Capillary Electrophoresis in an ABI Genetic Analyzer (Figure 2.3.2.).

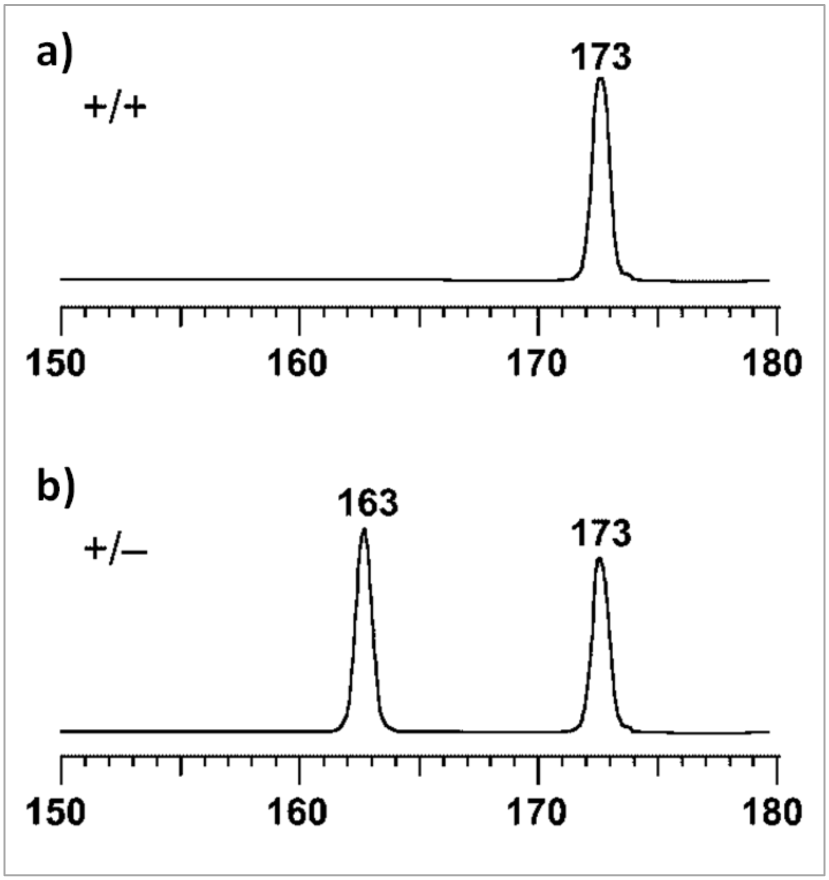


Figure 2.3.2. Illustration of sex identification methodology by using PCR with a labeled primer and ABI Capillary Electrophoresis. In a chromatograph: a) an homozygous individual is expected to have a single peak , b) whereas an heterozygous individual is expected to have two peaks, indicating the two different copies of the targeted gene (Wilhelm *et al*. 2002).

For the amplification of the samples by PCR, the same protocol as written in Chapter 2.3. was performed, with the exception that a 6-FAM labeled forward 1237L primer was used instead. Subsequent to PCR, 9 μL of a mix including Hi-Di™ Formamide and GeneScan™ 500 LIZ® Size Standard were added to each sample prior to their denaturation in a thermal cycler for 7 min at 95^{o}C. The analysis of the labeled PCR products was conducted in an ABI Genetic Analyzer and allele scoring was performed using STRand Analysis Software.

2.4. Statistical analyses

Statistical analyses were performed in R studio 4.0.3. Maps were designed using ArcGIS Pro 2.7.2. Software.

The data were collected during three different time periods, thus they refer to three different bird developmental stages: i) the stage of the egg, ii) the stage of the nestling, iii) the stage of the fledgling. Due to the fact that within years some of the eggs could not survive until fledging, the data analysis was split into three parts: i) **with - losses analysis**, where the whole dataset was analyzed including the nests in which mortality had occurred between the stage of the egg and the time of ringing, ii) **full – clutch analysis**, where only the nests with no mortality from the stage of the egg until the stage of the fledgling were analyzed, and iii) **full – brood analysis**, where only the nests with no mortality from the stage of the nestling until the stage of the fledgling were analyzed.

Data exploration

The first step in the analysis process was the exploration of the data by using descriptive statistics and drawing inferences for all of the variables included in this study.

The **sex ratio** was calculated as the proportion of males. Since the sex data originated from the stage of fledging chicks, results related to the proportion of sexes at the time of birth, namely to the secondary sex ratio (Mayr 1939).

For each fledgling the **age** since hatching was estimated, following the method that was described by Ristow & Wink, 2004 (2.4.1.).

$$A = \frac{\mathrm{WC}+52.1}{9.71} \quad \text{for } 45 < WC < 113 \quad \text{and}$$

$$A = \frac{\mathrm{WC}-8.3}{6.16} \quad \text{for } 113 < WC < 242$$

(Equation 2.4.1)

A = age of fledgling in days

WC = wing chord of fledgling in mm

Once the age of each individual was assessed, its **hatching date** was calculated by subtracting its age from the date that the bird was ringed. By estimating the range and the average age for all fledglings, it was found that individuals could be split into three different hatching dates: i) those who were hatched before 24/8 (early), ii) those who were hatched on the 24/8 (early – late), and iii) those who were hatched after 24/8 (late).

Besides the age and the hatching date for each chick, the **hatching order or rank** was estimated. Since the maximum clutch size that Eleonora's falcon females can produce is three eggs, the rank was classified into three levels: i) rank 1, ii) rank 2, and iii) rank 3. The rank was initially assessed by direct observations and measurements of fledglings on the field, with the heavier and bigger in size chicks to be the first to hatch, and it was later verified with the age equation (2.4.1.).

Nest quality for each nest was estimated by using an equation which takes into account three different variables: the sector of the island in which each nest is located (Sector), the type of the nest (Nest type) and the orientation of the nest in the island (Orientation). The equation that calculates the nest quality for each nest of the islet is being described below (2.4.2.):

$$\boxed{\text{Nest quality} = (0.25*\text{Sector}) + (0.5*\text{Nest type}) + (0.25*\text{Nest orientation})}$$

(Equation 2.4.2.)

Sector is a factor with 5 levels (a, b, c, d, e), where each level corresponds to a different part of the island and is described by a specific value (sector index): a=1, b=3, c=2.5, d=2, e=4. The different sectors reflected the microclimatic differences that exist in the islet of Anavatis and were categorized as follows: a = humid and hot, b = hot and dry, c = hot, d = humid and cool, e = dry and cool.

Nest type is a factor with 7 levels (a, b, c, d, e, f, i), where each level reflects a different type of nest and is described by a specific value (a=1.5, b=2.5, c=3, d=2, e=4, f,i=3.5). Nest types were categorized as follows: a = bush, b = under boulder, c = ledge, d = pothole, e = inaccessible pothole/ledge/cave, f = accessible cave, h = open ground and i = burrow.

Nest orientation is a factor that describes the direction or bearing of each nest.

Clutch size refers to the number of eggs laid within a nest, whereas **brood size** refers to the number of nestlings within a nest.

Statistical models

To assess the influence of specific factors on sex allocation, generalized linear mixed models (GLMMs) with a binomial error distribution (logit – link function) and year as a random factor were constructed in the R package glmmTMB. Number of females among the number of chicks was chosen as the binomial response variable together with the following explanatory variables: type index, sector index, orientation, rank, age and hatching date. To select the model with the best fit of the data, a series of step-wise deletion GLMM tests was applied and any non significant explanatory variables, factors and interaction terms were removed, resulting in the minimal model. The best model was the one with lowest Akaike Information Criterion (AIC) and levels of significance (p-values) for the variables included in each model were, also, taken into account. Best models were also tested for overdispersion and collinearity.

2.5. Sexual weight dimorphism of fledglings

To assess the weight dimorphism between sexes the difference in average body mass (gr) at ringing between females and males was calculated. Body mass was also expressed in relation to rank and brood size in order to address the size dimorphism within a nest, as well as in relation to hatching date to address any potential effect of the season on body mass between sexes.

3. Results

3.1. Molecular sexing

The aim of this study was, first, the sex identification of Eleonora's falcon offspring by using molecular sexing techniques. DNA was extracted successfully from 535 fledglings and the sex of each individual was identified with PCR. The samples that had two bands on a 3% agarose gel were characterized as females and the single band samples as males (Figure 3.1.1.).

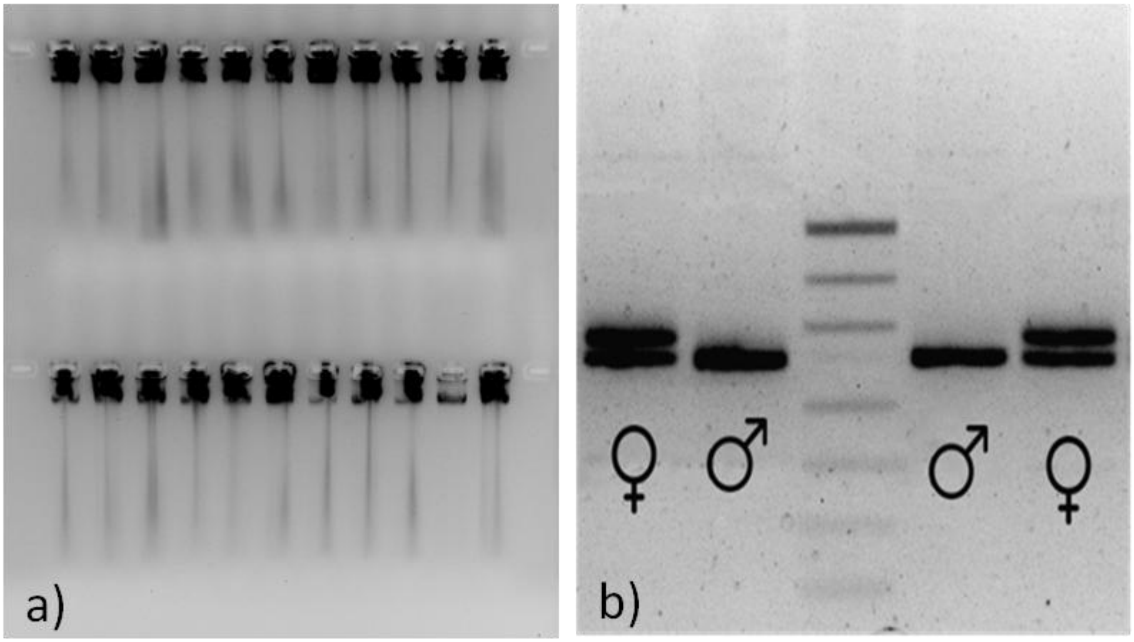


Figure 3.1.1. DNA extraction and PCR amplification for Eleonora's falcon. a) total DNA bands on a 1.5 % agarose gel, b) PCR products on a 3 % agarose gel. The double bands indicate a female individual, whereas the single band a male.

To verify the reliability of the gel – based sex identification method, 50 samples for which the sex was already identified, were later analyzed by genotyping. All males had a single peak at 296 bp, while all females had two peaks, at 296 bp and 318 bp, in a chromatograph (Figure 3.1.2).

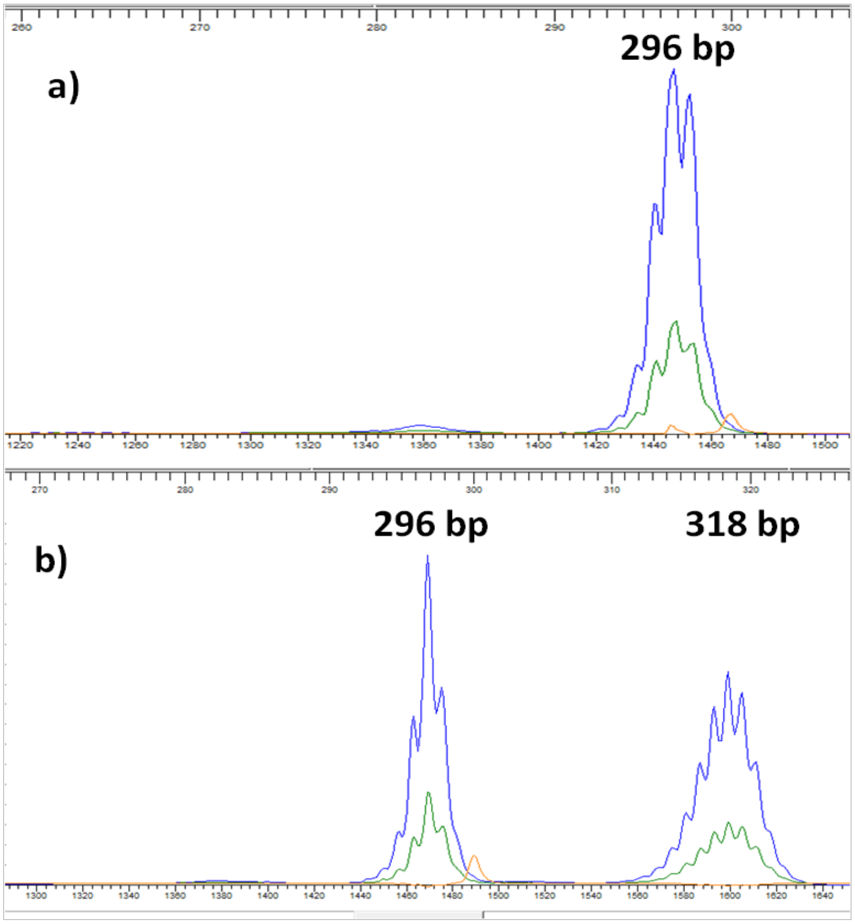


Figure 3.1.2. Chromatographs of an ABI Capillary Electrophoresis for the CHD1 gene. a) A male individual has two CHD1 fragments of 296 bp each, resulting in a single peak, b) A female individual has two CHD1 fragments: a 296 bp fragment and a 318 bp one, resulting in two peaks in a chromatograph

3.2. Statistical analyses

Having obtained the sex for all 535 Eleonora's falcon fledgings, offspring sex ratio (proportion of males) was associated with a number of ecological and biological parameters, both on the individual and the family (nest) level (Table 3.2.1.).

Table 3.2.1. Summary of descriptive statistics for all the variables included in the data analysis.

Variables	Mean ± SD	Range	Levels*
Year	-	2009-2020	12
Nest	-	1-150	98
Sector	-	a-e	5
Type	-	a-i	7
Sector Index	-	1-4	5
Type Index	-	1.5-4	6
Orientation	-	3-358	86
Nest quality	2.81 ± 0.64	1.25-4	18
Sex	-	0-1	2
Rank	-	1-3	3
Age (days)	26.10 ± 4.18	10-37	-
Hatching date	-	early-late	3
535 observations in total			

Data represent the Mean and Standard Deviation (SD) and the Range (Min – Max) for each variable.

* **Levels** describe the number of levels that each categorical variable has.

i) With – losses analysis

In total, 98 distinct nests (259 in total) were visited and sampled during 12 consecutive years (2009 – 2020), resulting in 535 individuals. No blood or feather samples were collected from adult birds. For this reason, all of the results that are presented below refer to young chicks (10 – 37 days old).

Year and sex

In total, out of 535 individuals for which the sex was identified, 268 were males and 267 were females, resulting in an overall sex ratio of 0.51 and an average sex ratio per year of 0.52 ± 0.08. Sex ratio was 8 out of 12 years male biased, whereas during the years with the highest number of offspring - between 2015 and 2017 – and during the year with the lowest number of offspring – 2009 – females outnumbered males. Although fledglings' annual sex ratios were found to deviate from parity, the proportion of sexes both within and between years did not differ statistically significantly (X^2 (11, N = 535) = 11.03, p = .44; Chi-squared test for each year resulted in p > .05) (Figure 3.2.1.; Table 3.2.2.)

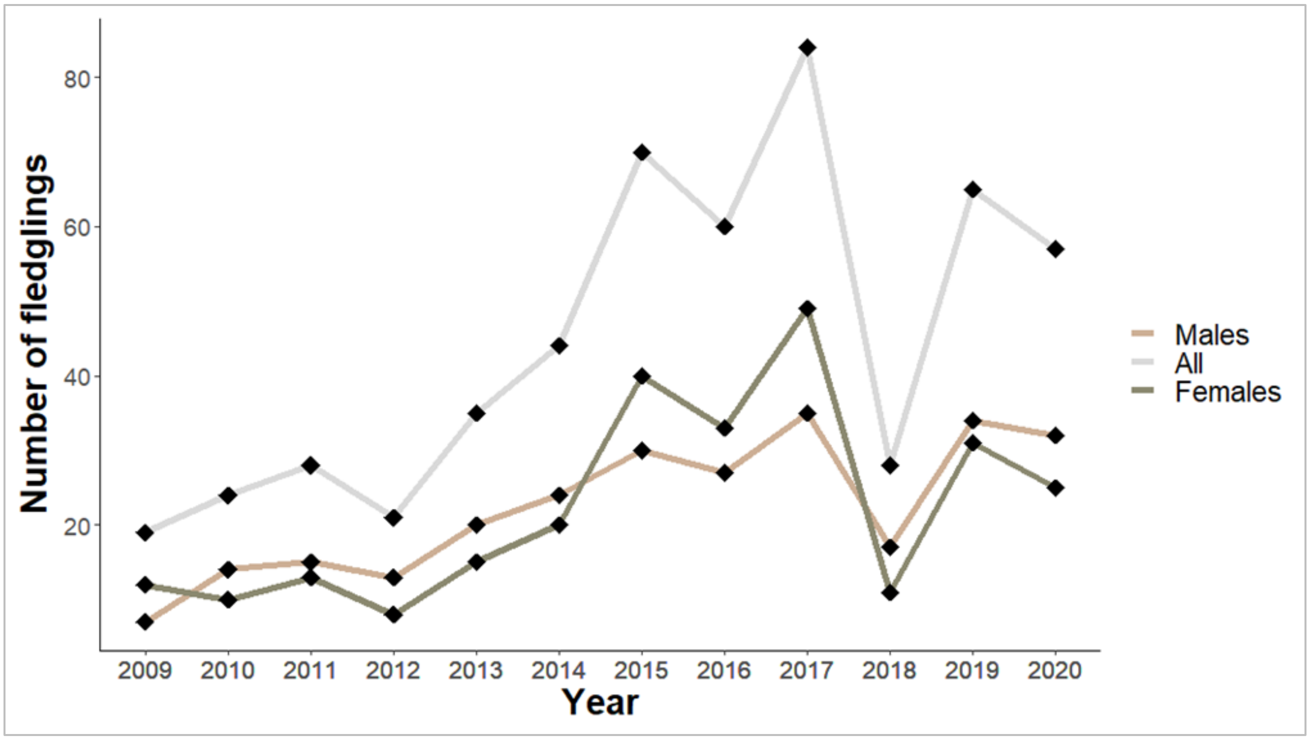


Figure 3.2.1. Fluctuations in the number of Eleonora's falcon offspring over years

Table 3.2.2. Number and proportion of fledglings over years

Year	Females	Males	Total	Sex ratio
2009	12	7	19	0.37
2010	10	14	24	0.58
2011	13	15	28	0.54
2012	8	13	21	0.62
2013	15	20	35	0.57
2014	20	24	44	0.55
2015	40	30	70	0.43
2016	33	27	60	0.45
2017	49	35	84	0.42
2018	11	17	28	0.61
2019	31	34	65	0.52
2020	25	32	57	0.56

Rank and sex

Next, sex ratios were analyzed in relation to the chicks' hatching order within a brood. In contrast with the non - significant differences in the sex ratio between years, it was found that the sex ratio between ranks was obviously biased. The proportion of sexes differed statistically significantly between ranks (X^2 (2, N = 535) = 15.18, $p < .001$), implying that within family, there was a significant inclination towards females in the first – rank chicks and towards males in the chicks hatched second or third within a brood (Figure 3.2.2.). The sex ratio was 0.41 for rank 1 and 0.56 and 0.60 for rank 2 and 3 respectively. In addition, the rank – related sex ratios were calculated for each year separately (Table 3.2.3.). The sex ratio bias pattern described above was consistent for most of the years.

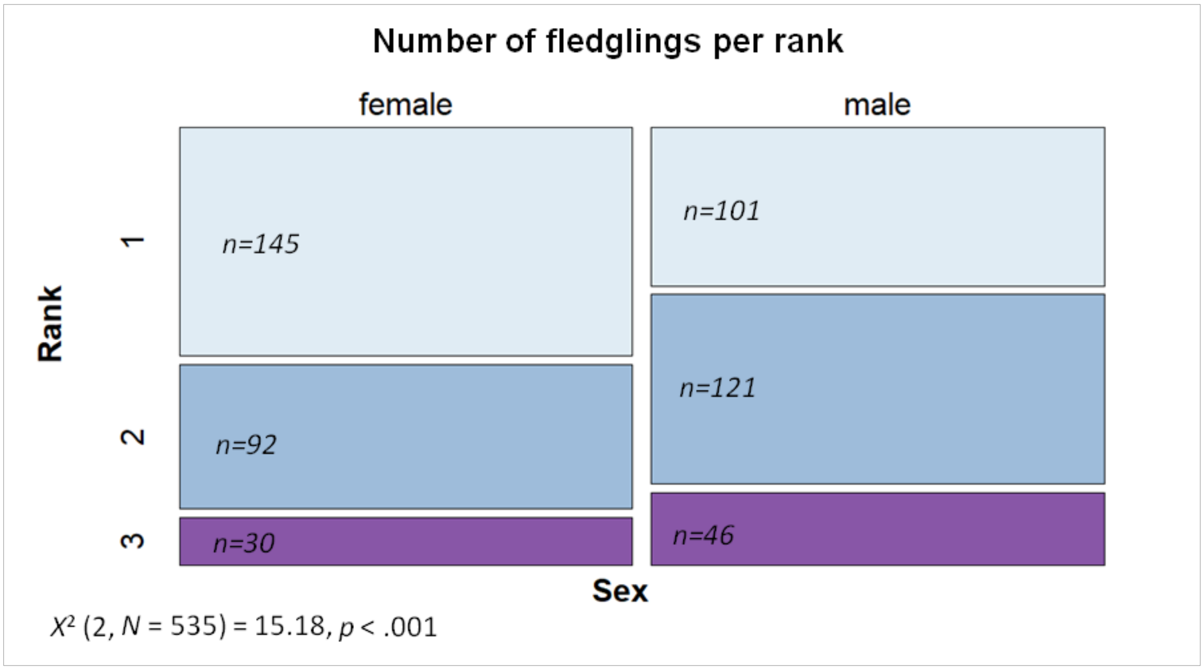


Figure 3.2.2. Proportions of sexes between different ranks.

Table 3.2.3. Sex ratio (proportion of males) in relation to rank and year.

	Sex ratio		
Year	Rank 1	Rank 2	Rank 3
2009	0.30	0.43	0.50
2010	0.55	0.50	1.00
2011	0.42	0.64	0.60
2012	0.58	0.57	1.00
2013	0.50	0.60	1.00
2014	0.40	0.76	0.43
2015	0.37	0.48	0.50
2016	0.40	0.48	0.50
2017	0.32	0.47	0.58
2018	0.50	0.73	0.67
2019	0.35	0.64	0.64
2020	0.48	0.64	0.60

Hatching date and sex

A similar pattern with rank was identified when the relationship of the sex ratio with the hatching date of fledgling was investigated. Chicks had an average age of 26.10 ± 4.18 days, with the age to vary between 10 and 37 days. Female average age was 27.07 ± 4.17 days, with a range of 12 to 35 days, whereas males had a lower average age of 25.14 ± 3.96 days and a range of 10 to 34 days. The proportion of females was statistically significantly higher than the proportion of males for the early - hatched chicks and significantly lower for the late - hatched ones (X^2 (2, N = 535) = 19.02, p < .001) (Figure 3.2.3.). As a consequence, individuals that were hatched early in the breeding season were more likely to be females, whereas the individuals that were hatched late in season were more likely to be males. The sex ratio was 0.41 for early hatched chicks and 0.61 and 0.52 for the late and early – late hatched chicks respectively. Additionally, the hatching date – related sex ratios were calculated for each year separately (Table 3.2.4.). The observed seasonal bias was found to be consistent in most of the years.

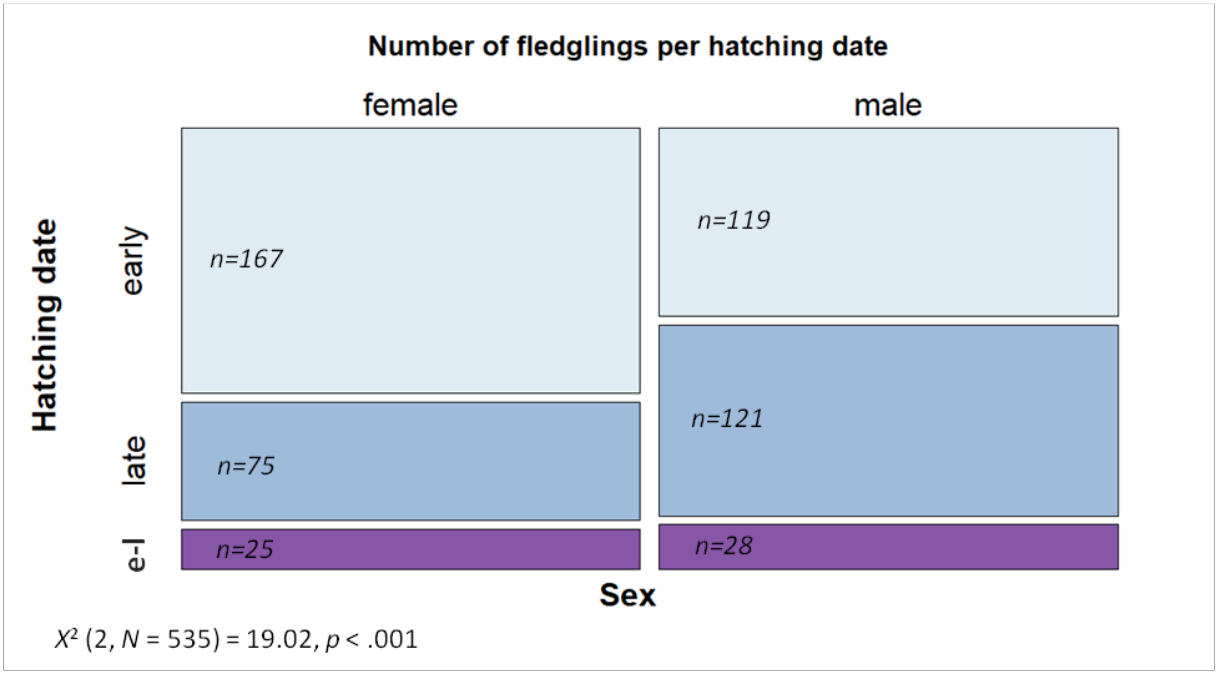


Figure 3.2.3. Proportions of sexes between different hatching dates.

Table 3.2.4. Sex ratio (proportion of males) in relation to hatching date and year.

		Sex ratio	
Year	Early	Early - Late	Late
2009	0.00	0.00	0.39
2010	0.14	1.00	0.75
2011	0.00	0.00	0.56
2012	0.17	0.80	0.80
2013	0.52	0.43	0.86
2014	0.50	0.00	0.67
2015	0.33	0.71	0.64
2016	0.33	0.57	0.71
2017	0.38	0.25	0.52
2018	0.57	0.00	0.69
2019	0.51	0.63	0.50
2020	0.48	0.50	0.70

Nest quality, sector, nest type, orientation and sex

The sex ratio in relation to the nest quality and the nest quality parameters such as sector, nest type and orientation was first analyzed. A one-way ANOVA was performed to evaluate if the nest quality was different for the two sexes. Nest quality was not statistically significantly different between sexes (ANOVA (F (1,535) = 0.551, p = .458) (Appendix 2; Figure 1; Table 1).

However, when sector, nest type and orientation were separately analyzed the results were different. A chi-square test of independence was performed to examine the relationship between sex and sector, nest type or orientation. The frequencies of females and males differed significantly between sectors (X^2 (4, N = 535) = 10.80, p = .028) (Table 3.2.5; Figure 3.2.4.) and orientation, (X^2 (14, N = 535) = 26.06, p = .025) (Figure 3.2.5.), but not between different nest types, (X^2 (6, N = 535) = 4.05, p = .67) (Appendix 2; Table 2). In conclusion, proportion of sexes was more likely to differ between sectors and nest orientation, than between different types of nest.

Table 3.2.5. Number and proportion of fledglings in relation to sector

Sector	Females	Males	Total	Sex ratio
a	37	42	79	0.53
b	22	30	52	0.57
c	32	54	86	0.62
d	74	60	134	0.44
e	102	82	184	0.44
Total	**267**	**268**	**535**	

X^2(4, N = 535) = 10.80, ***p = .028*** *significant differences between sectors*

A Kernel Density map was drawn to illustrate the differences in the proportion of sexes between different sectors in the island (Figure 3.2.4.). Despite the small surface of the islet, there are some microclimatic differences between sectors and this was seen to affect the proportion of the sexes. Nests that belong to the sectors e and d, that both provide a cooler habitat, were the ones with higher percentages of females. Conversely, the nests that belong to the sectors a, b and c, were more exposed to heat and had higher number of males.

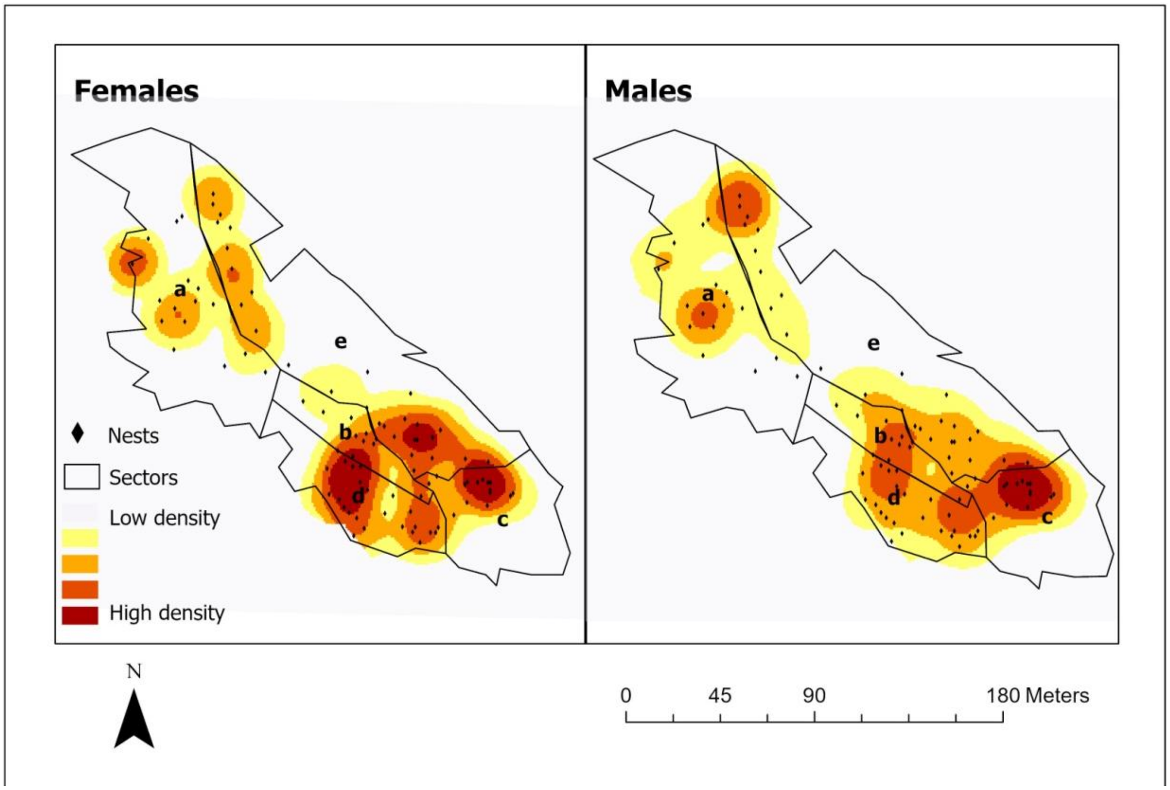


Figure 3.2.4. Proportion of females and males fledglings in different sectors: a = humid and hot, b = hot and dry, c = hot, d = humid and cool, and e = dry and cool. The darker colors represent the higher percentage of fledglings.

The effect of the nest orientation on the sex ratio was graphically explained by plotting the percentage of males in relation to the orientation of the nest (Figure 3.2.5.). The highest percentage of males was found in SE (135^{o}) – facing and SW (225^{o}) or WNW (270 – 300^{o}) – facing nests. In contrast, more females were born in nests with an eastern (90 - 100^{o}) orientation, as well as in those facing SSW (200 ^{o}). These results are in accordance with those obtained when sex ratio was compared with the sector, where the percentage of female chicks was higher in the eastern and south-southeastern part of the island.

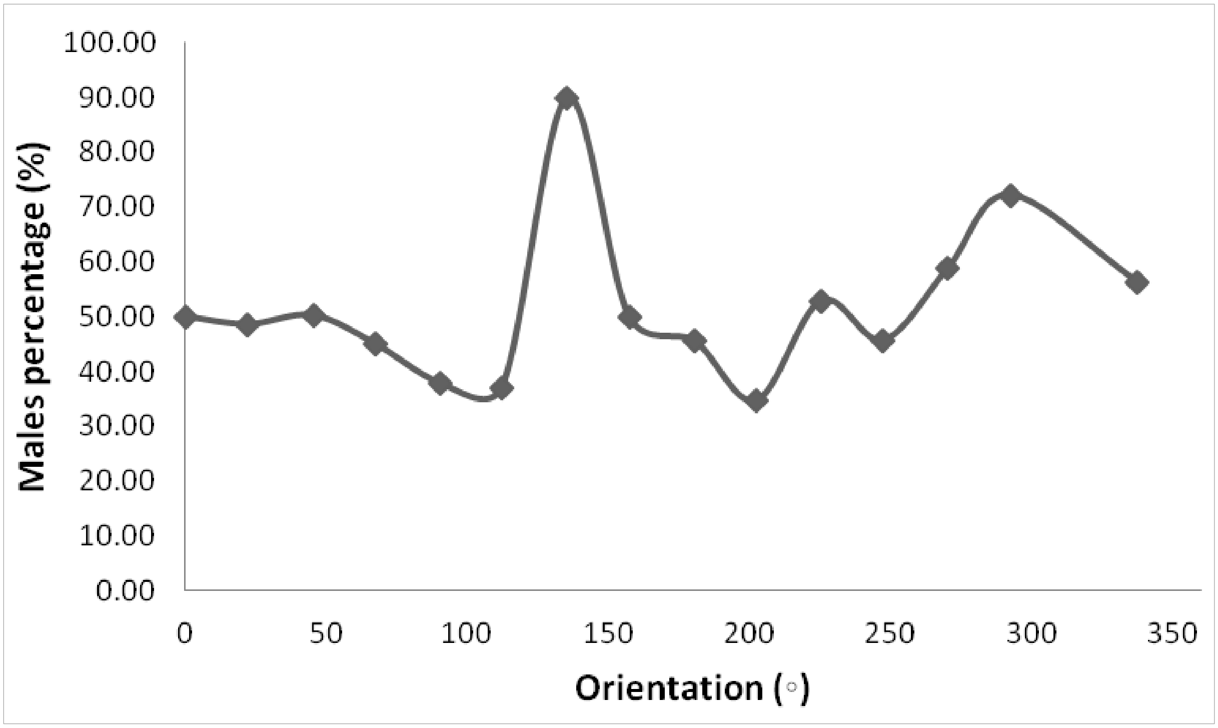


Figure 3.2.5. Proportions of females and males fledglings in relation to the orientation of the nest

Statistical models

In the first part of the analysis each parameter was examined separately for any potential effect that it might have on the offspring sex ratios. The next step in the analysis was to combine all of the variables that were explored in the first part and investigate their cumulative effect on the sex ratio by constructing generalized linear mixed models (GLMMs). In these GLMMs, individual fledgling sex (probability to be a male) was the response variable and explained by the following fixed variables: i) nest type index, ii) sector index, iii) nest orientation, iv) rank, vi) age and vii) hatching date. Consecutive models were designed (Table 3.2.6.) and the best model was selected according to the Akaike Information Criterion (AIC) (Appendix 2, Table 3 - Table 7) and the significance of the variables' p-value.

Table 3.2.6. Summary of the GLMM models that were designed in the with-losses analysis.

Model	Type index	Sector index	Orientation	Rank	Age	Hatching date	Random factor	AIC	BIC
#1	✔	✔	✔	✔	✔	✔	Year	713.9	752.5
#2			✔	✔	✔		Year	709.3	730.8
#3				✔	✔		Year	712	729.1
#4				✔*	✔*		Year	713.2	734.7
#5			✔	✔	✔		Year|Nest	NA	NA
#6			✔	✔	✔		Nest	710.5	731.9

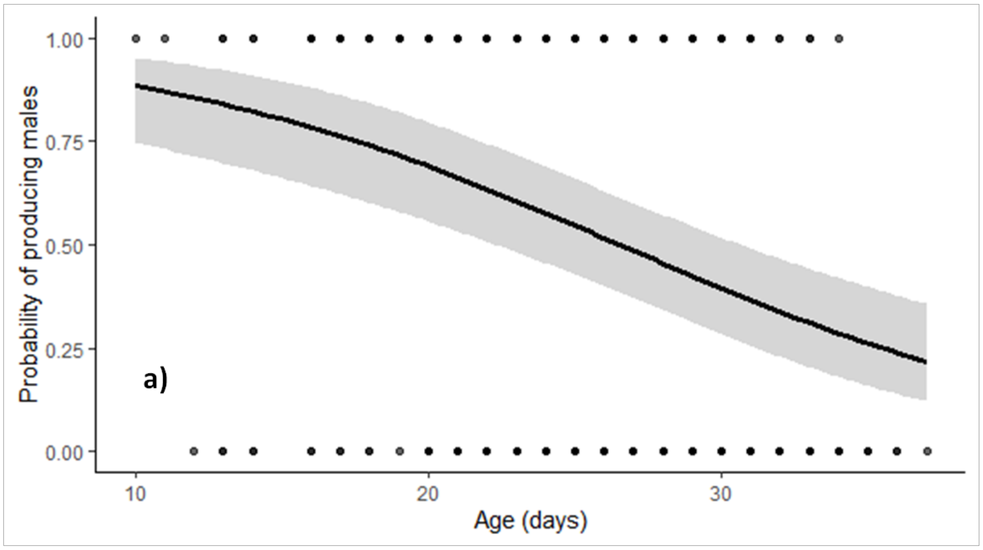
*Interaction between Age and Rank

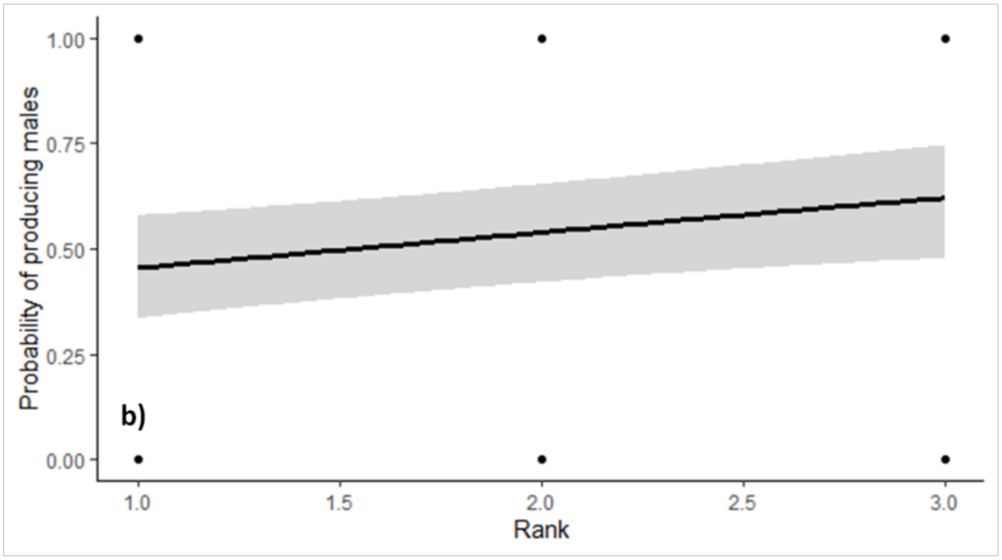
Out of all variable combinations, the final model included three explanatory variables such as orientation, rank and age (Table 3.2.7.). No significant interactions were found between fixed factors, thus only main effects were included in the final model. The probability of the sex to be male increased with orientation (Estimate = 2.353 ± 0.734) and rank (Estimate = 0.337 ± 0.001) and decreased with age of fledgling (Estimate = -0.122 ± 0.025) (Figure 3.2.6.). Age had the strongest effect on predicting the sex of an offspring ($p<.001$). Sector and hatching date could not significantly predict the expected outcome, since sector's effect was overshadowed by orientation and hatching's date by age. In conclusion, GLMM produced the best combination of parameters for predicting the sex ratio in Eleonora's falcon fledglings, namely the hatching order (rank), the age and the orientation of the nest.

Table 3.2.7. Results of the GLMM model with the best combination of predictors on estimating the probability of the sex to be male in Eleonora's falcon fledglings (With – losses analysis).

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	2.353	0.734	3.205	0.001 **
Orientation	0.002	0.001	2.138	0.033 *
Rank	0.337	0.132	2.556	0.011 *
Age	-0.122	0.025	-4.868	<.01 ***
	Variance	**Standard Deviation**		
Year	0.044	0.209		
AIC	709.3			

*Significance codes: 0 '***' , 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ',' 1*





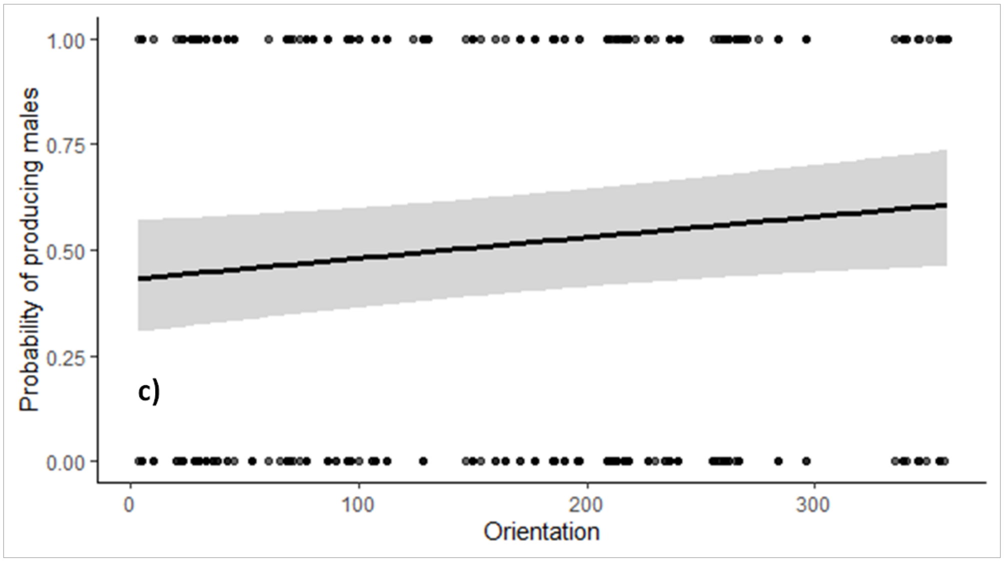


Figure 3.2.6. Relationship between the response variable ‘Sex’ (probability of producing males) and the predictor variables: a) ‘Age’, b) ‘Rank’, c) ‘Orientation’.

ii) Full - clutch analysis

To verify the results that were obtained so far, the same analyses were repeated for the subset of nests with no mortality from the stage of the egg until the stage of the fledgling (full – clutch nests). Since this subset of data excludes any missing information caused by the mortality of some chicks between different developmental stages, the full – clutch analysis was expected to provide the most realistic results regarding the offspring sex ratio patterns.

In total, the full – clutch analysis included 70 distinct nests (150 in total), resulting in 362 chicks, out of which 184 were females and 178 were males. The overall sex ratio (proportion of males) was 0.49, while the average annual sex ratio was 0.58 ± 0.079 (Appendix 2, Table 8). Although fledglings' annual sex ratios were found to deviate from parity, the proportion of sexes both within and between years were not statistically significant (X^2 (11, N = 362) = 14.55, p = .20; 2 – proportions z-test for each year resulted in p > .05).

In accordance with the initial data analysis, the sex ratio was significantly female biased on the first rank (Sex $\text{ratio}_{\text{Rank 1}}$ = 0.36) and male biased on the second (Sex $\text{ratio}_{\text{Rank 2}}$ = 0.57) and third rank (Sex $\text{ratio}_{\text{Rank 3}}$ = 0.63) (X^2 (2, N = 362) = 18.47, p < .001). Similarly, the sex ratio was significantly female biased on the early - hatched group of chicks (Sex $\text{ratio}_{\text{Early}}$ = 0.38) and male biased on the late (Sex $\text{ratio}_{\text{Late}}$ = 0.66) and early – late (Sex $\text{ratio}_{\text{Mean}}$ = 0.53) - hatched chicks (X^2 (2, N = 362) = 23.83, p < .001). Annual variation of sex ratios in relation to rank and hatching date were found to be consistent in most of the years (Appendix 2, Table 9-Table 10). In contrast with the analysis where all nests were included, nest quality, nest type, sector and orientation had no significant effect on the sex ratio.

Additionally, since the full information of the clutch was available in this subset of the dataset, the sex ratios in relation to the clutch size and the hatching date were calculated (Table 3.2.8). The results indicated that early in season the number of 3-egg-clutches, as well as 2-egg-clutches was higher and more females were produced during that time (Sex $\text{ratio}_{\text{early, clutch = 3}}$ = 0.36; Sex $\text{ratio}_{\text{early, clutch = 2}}$ = 0.41). The opposite pattern, hence a higher proportion of males, was observed for the late - hatched clutches (Sex $\text{ratio}_{\text{late, clutch = 3}}$ = 0.70; Sex $\text{ratio}_{\text{late, clutch = 2}}$ = 0.62).

Table 3.2.8. Proportion of fledglings in relation to clutch size and hatching date

Hatching date		Clutch size = 1	Clutch size = 2	Clutch size = 3
-	N	5	156	201
	Sex ratio	0.6	0.49	0.49
	Nests	*5*	*78*	*67*
Early	N	2	75	127
	Sex ratio	0.5	0.41	0.36
Late	N	2	65	61
	Sex ratio	0.5	0.62	0.7
Early - Late	N	1	16	13
	Sex ratio	1	0.38	0.69

A series of GLMMs were, later, constructed in an attempt to investigate those factors that can best explain the sex ratio of Eleonora's falcon offspring (Table 3.2.9.; Appendix 2: Table 11-Table 12). In the same way as the previous GLMM analysis, when offspring mortality wasn't taken into account, the final model included three explanatory variables such as orientation, rank and age. No significant interactions were found between fixed factors, thus only main effects were included in the final model. The probability of the sex to be male increased with orientation (Estimate = 0.003 ± 0.001) and rank (Estimate = 0.435 ± 0.160) and decreased with age of fledgling (Estimate = -0.146 ± 0.032) (Table 3.2.10.).

Table 3.2.9. Summary of the GLMM models that were designed in the full - clutch analysis.

Model	Type index	Sector index	Orie - ntation	Rank	Age	Hatching date	Clutch size	Random factor	AIC	BIC
#1	✔	✔	✔	✔	✔	✔	✔	Year	472.1	511.1
#2			✔	✔	✔	✔	✔	Year	468.8	500.0
#3			✔	✔	✔			Year	465.2	484.7

Table 3.2.10. Results of the GLMM model with the best combination of predictors on estimating the probability of the sex to be male in Eleonora's falcon fledglings (Full – clutch analysis).

Variables	Estimates	Standard Error	Z – value	P
Intercept	2.490	0.946	2.632	0.008 **
Orientation	0.003	0.001	3.074	0.002 **
Rank	0.435	0.160	2.711	0.007 **
Age	-0.146	0.032	-4.530	<.01 ***
	Variance	**Standard Deviation**		
Year	0.119	0.345		
AIC	465.2			

*Significance codes: 0 '***' , 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ,' 1*

iii) Full - brood analysis

Finally, the subset of nests without any mortality from the stage of the nestling until the stage of the fledgling (full – brood nests) was analyzed.

In total, 87 distinct nests (217 in total) were examined, resulting in 468 chicks, out of which 224 were females and 244 were males. The overall sex ratio (proportion of males) was 0.52, while the average annual sex ratio was 0.54 ± 0.088 (Appendix 2, Table 13). Although fledglings' annual sex ratios were found to deviate from parity, the proportion of sexes both within and between years were not statistically significant (X^2 (11, N = 468) = 9.63, p =.30; 2 – proportions z-test for each year resulted in p > .05).

In line with the previous results, the sex ratio was significantly female biased on the first rank (Sex $\text{ratio}_{\text{Rank 1}}$ = 0.42) and male biased on the second (Sex $\text{ratio}_{\text{Rank 2}}$ = 0.60) and third rank (Sex $\text{ratio}_{\text{Rank 3}}$ = 0.63) (X^2 (2, N = 468) = 15.55, p < .001). Furthermore, the sex ratio was significantly female biased on the early - hatched group of chicks (Sex $\text{ratio}_{\text{Early}}$ = 0.43) and male biased on the late (Sex $\text{ratio}_{\text{Late}}$ = 0.64) and early – late (Sex $\text{ratio}_{\text{Mean}}$ = 0.56) - hatched chicks (X^2 (2, N = 362) = 18.89, p < .001). Annual variation of sex ratios in relation to rank and hatching date were consistent in most of the years (Appendix 2, Table 14; Table 15). Only between sectors the proportions of sexes were found to vary statistically significantly (X^2 (4, N = 468) = 9.63, p = .04), whereas no difference in the proportions was identified in relation to nest quality, nest type and orientation.

Larger broods tended to start earlier in the breeding season ($N_{early, brood = 3}$ = 127;$N_{early, brood = 2}$ = 109) and those broods were female biased (Sex $ratio_{early, brood = 3}$ = 0.36; Sex $ratio_{early, brood = 2}$ = 0.48). Contrarily, larger broods were less abundant later in the season ($N_{late, brood = 3}$ = 61; $N_{late, brood = 2}$ = 98) and were male biased (Sex $ratio_{late, brood = 3}$ = 0.70; Sex $ratio_{late, brood = 2}$ = 0.59) (Table 3.2.11).

Table 3.2.11. Proportion of fledglings in relation to brood size and hatching date

Hatching date		**Brood size = 1**	**Brood size = 2**	**Brood size = 3**
-	N	44	232	201
	Sex ratio	0.71	0.52	0.49
	Nests	*34*	*116*	*67*
Early	N	13	109	127
	Sex ratio	0.61	0.48	0.36
Late	N	17	98	61
	Sex ratio	0.71	0.59	0.7
Early - Late	N	5	25	13
	Sex ratio	1	0.4	0.69

The expected sex was explained by GLMMs and the best model was selected amongst a series of others (Table 3.2.12.; Appendix 2: Table 16-Table 18). The final model included four explanatory variables, such as orientation, rank, age and brood size. No significant interactions were found between fixed factors, thus only main effects were included in the final model. The probability of the sex to be male increased with orientation (Estimate = 0.002 ± 0.001) and rank (Estimate = 0.569 ± 0.162) and decreased with age of fledgling (Estimate = -0.103 ± 0.028) and brood size (Estimate = - 0.501 ± 0.186) (Table 3.2.13; Figure 3.2.8.). The additional information that this model provided compared to the previous analyses was the contribution of brood size on the prediction of sex. As a result, the larger brood was more likely to produce more females.

Table 3.2.12. Summary of the GLMM models that were designed in the full – brood analysis.

Model	Type index	Sector index	Orie-ntation	Rank	Age	Hatching date	Brood size	Random factor	AIC	BIC
#1	✔	✔	✔	✔	✔	✔	✔	Year	614.9	656.3
#2	✔	✔	✔	✔	✔		✔	Year	612.0	645.2
#3	✔		✔	✔	✔		✔	Year	610.6	639.6
#4			✔	✔	✔		✔	Year	611.3	636.2

Table 3.2.13. Results of the GLMM model with the best combination of predictors on estimating the probability of the sex to be male in Eleonora's falcon fledglings (Full – brood analysis).

Variables	Estimates	Standard Error	Z - value	P
Intercept	2.632	0.8	3.291	<.01 ***
Orientation	0.002	0.001	2.410	0.015 *
Rank	0.569	0.162	3.514	<.01 ***
Age	-0.103	0.028	-3.679	<.01 ***
Brood size	-0.501	0.186	-2.706	0.006**
	Variance	**Standard Deviation**		
Year	0.015	0.124		
AIC	611.3			

*Significance codes: 0 '***' , 0.001 '**', 0.01 '*', 0.05 '.', 0.1 ' ,' 1*

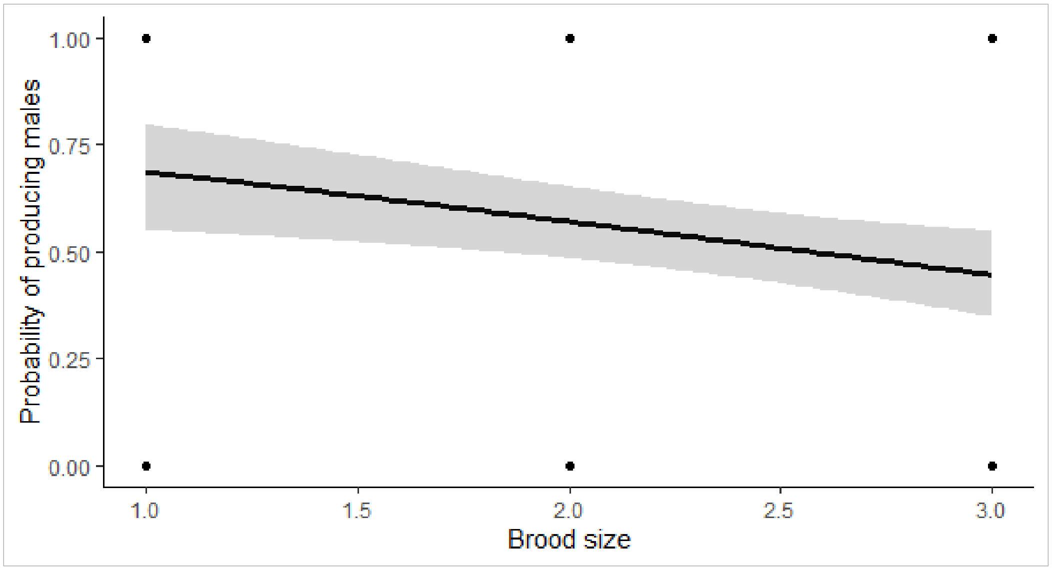


Figure 3.2.7. Relationship between the response variable 'Sex' (probability of producing males) and the predictor variable 'Brood size' when full – brood data were analyzed.

3.3. Sexual weight dimorphism of fledglings

The last objective of this study was to assess and quantify the sexual weight dimorphism that is known to occur in Eleonora's falcon, as well as identify external factors that might be related with differences in weight between sexes. For this reason, body mass was selected as an indicator of sexual weight dimorphism. The results demonstrated that all fledglings at the day of ringing had an average body mass of 456.93 ± 74.22 gr, ranging between 140 and 610 gr, with female fledglings to be by 50.17 ± 1.89 gr (11 %) heavier than males (Table 3.2.14.).

To avoid the bias in body mass that might have occurred due to the different age of the birds at the time of ringing, body mass was standardized by the age of each fledgling, hence body mass will correspond to body mass per age from now on. A non – parametric Kruskal – Wallis test was conducted to evaluate if the difference in body mass between females and males was statistically significant. Body mass was statistically significantly different between sexes *(*Chi squared = 34.6, p< .001, df = 1*)* (Table 3.2.15.; Figure 3.2.8.).

Table 3.2.14. Sexual weight dimorphism of fledglings in Eleonora’s falcon.

	Body mass (gr)		**Body mass/age (gr/day)**	
	Mean ± SD	Range	Mean ± SD	Range
Females	482.06 ± 68.92	210-610	17.89 ± 1.37	13.10 – 24.29
Males	431.89 ± 70.81	140-550	17.21 ± 1.56	12.73 – 22.11
Total	456.93 ± 74.22	140-610	17.55 ± 1.50	12.73 – 24.29

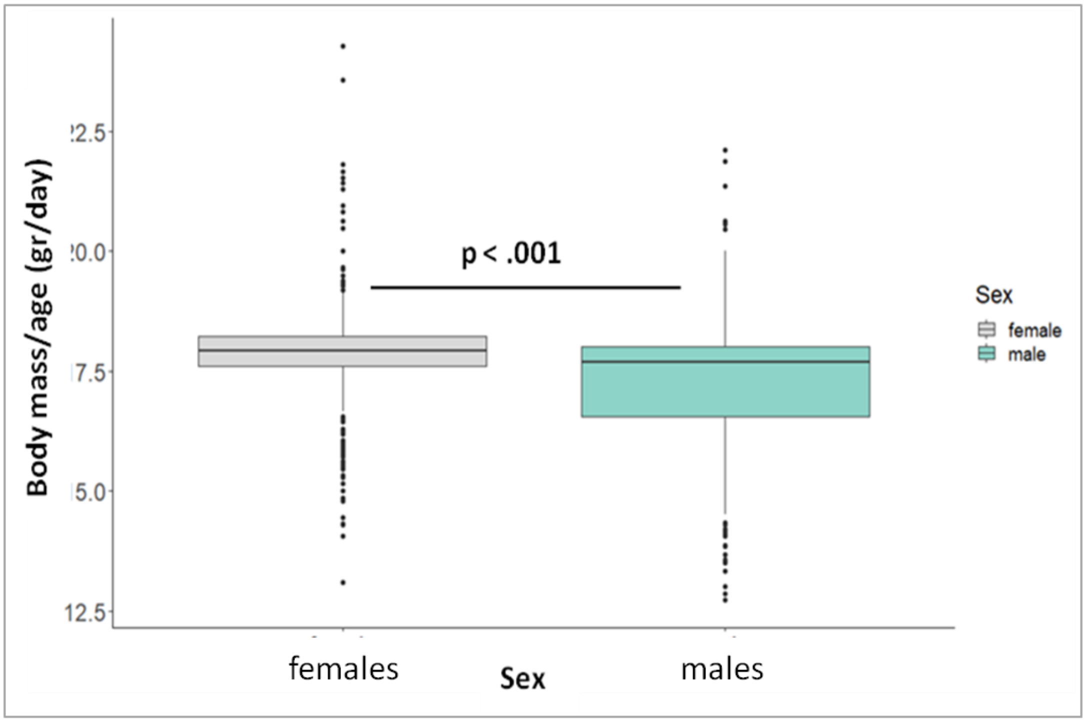


Figure 3.2.8. Body mass and sex. The p value indicates the significant difference in the mean body mass/age between sexes.

Differences in body mass were, also, described in relation to the hatching rank. The results showed no significant difference in average body mass between ranks for females (Kruskal – Wallis: (Chi squared = 1.21, p = .544, df = 2)). However, in males body mass varied significantly between first and third rank, but not between first and second rank (Kruskal – Wallis*:* (Chi squared = 6.29, p = .042, df = 2; Pairwise Wilcoxon test: (Rank 1 - Rank 3, p =. 048)). Thus, the third - rank male chick was significantly lighter than the first – rank one, a difference that was not observed in females. Additionally, for each rank body mass was found to vary significantly between sexes (Kruskal – Wallis: Rank1 (Chi squared = 11.63, p<.001, df = 1), Rank2 (Chi squared = 11.96, p<.001, df = 1), Rank3 (Chi squared = 6.95, p=. 008, df = 1) (Figure 3.2.9.). In conclusion, female fledglings were found to be significantly heavier than males in all ranks and male fledglings' body mass decreased with rank.

Next, the relationship between body mass and brood size was investigated. There were significant differences in average body mass between different brood sizes both in females (Chi-squared = 7.23, p = .026, df = 2; Wilcoxon pairwise test (Brood size=1- Brood size=3, Brood size=2- Brood size=3 : p = .06) and males (Chi-squared = 6.76, p = .03, df = 2; Wilcoxon pairwise test (Brood size=2- Brood size=3: p = .031), with body mass to decrease as brood size increases. Additionally, for each brood size body mass was found to vary significantly between sexes (Kruskal – Wallis: Brood size=1 (Chi squared = 6.91, p<.001, df = 1), Brood size = 2 (Chi squared = 16.21, p<.001, df = 1), Brood size = 3 (Chi squared = 13.46, p<.001, df = 1) (Figure 3.2.10.).

Lastly, body mass was explained in relation to the hatching date. No significant interactions were found between body mass and hatching date for both females and males. However, early and late in season body mass varied significantly between sexes (Kruskal – Wallis: Early (Chi squared = 18.67, p<.001, df = 1), Early - Late (Chi squared = 1,15, p=.03, df = 1), Late (Chi squared = 15.66, p<.001, df = 1) (Figure 3.2.11.).

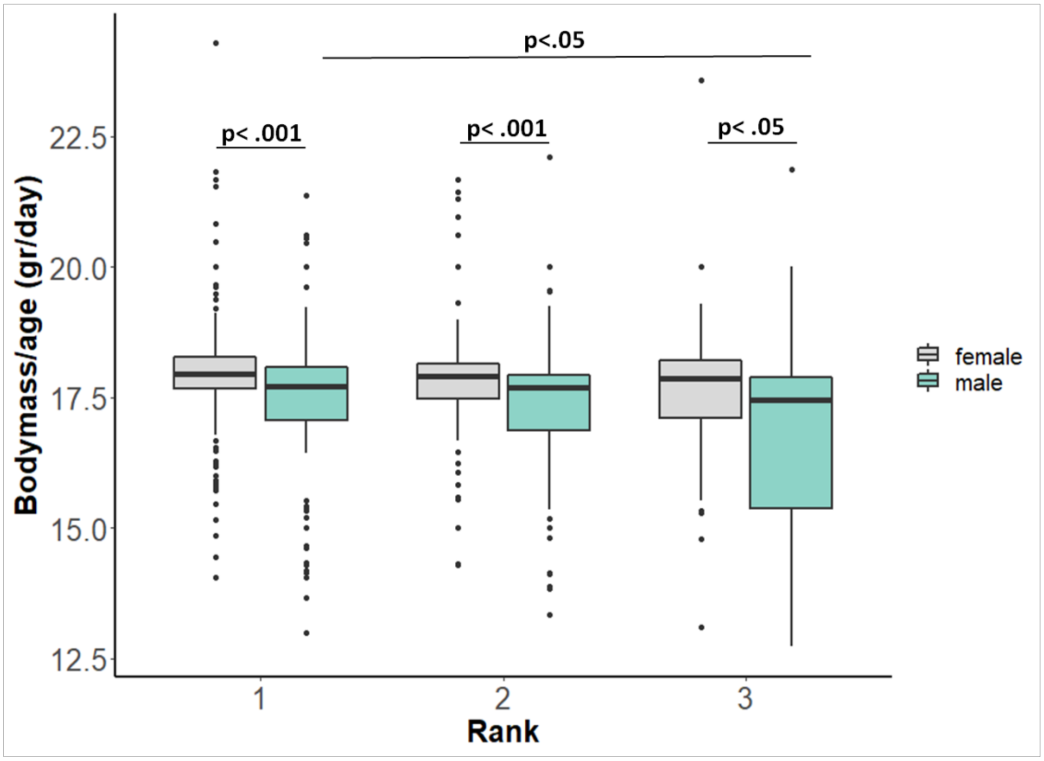


Figure 3.2.9. Body mass/age in relation to rank and sex.

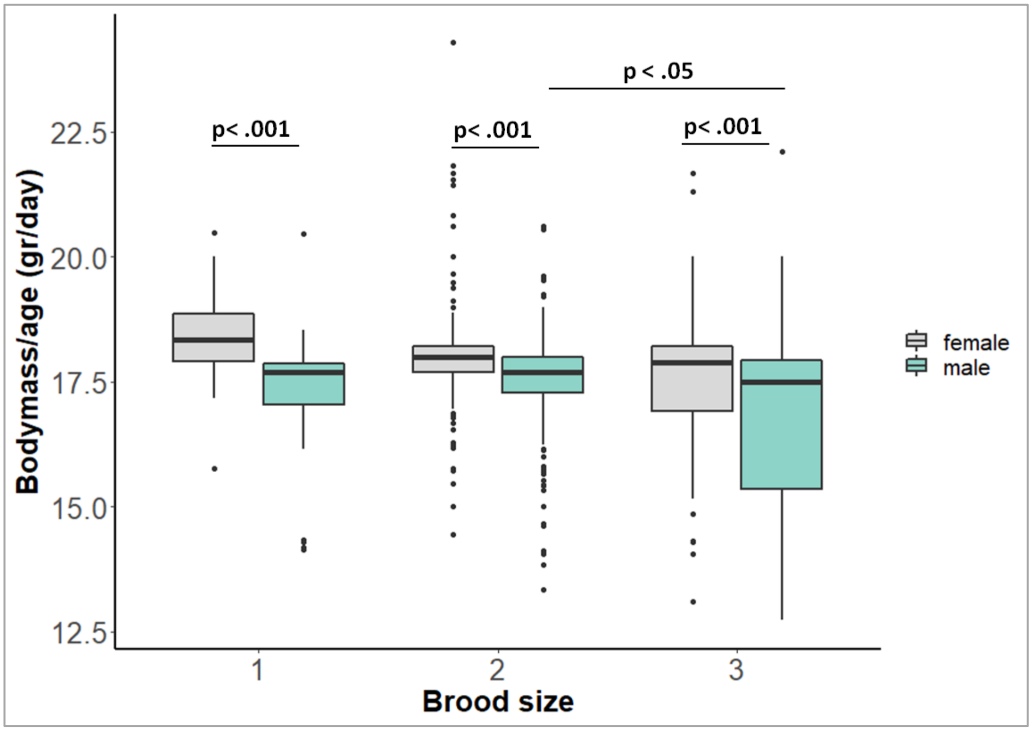


Figure 3.2.10. Body mass/age in relation to brood size and sex.

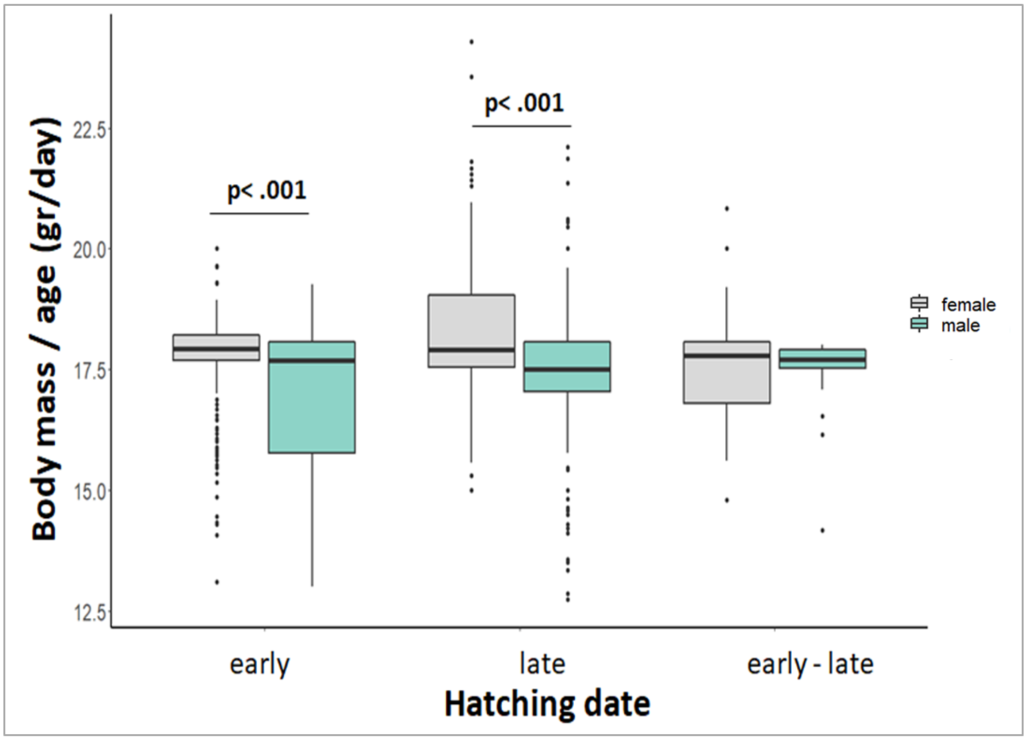


Figure 3.2.11. Body mass/age in relation to hatching date and sex.

4. Discussion

During this study the overall sex ratio (proportion of males) of Eleonora's falcon was slightly male biased (Sex ratio = 0.51). The sex ratio varied temporally, being significantly female-biased for rank 1 and early in the breeding season, and male-biased for rank 2 and 3, as well as late in the breeding season. Annual variation of the sex ratio was also observed despite being statistically insignificant. Additionally, the sex ratio varied with orientation of the nest, and brood size, with larger broods being female-biased. The same results were obtained for all three subsets of data: i) with losses from the stage of the egg until the stage of the fledgling, ii) without losses from the stage of the egg until the stage of the fledgling, and iii) without losses from the stage of the nestling until the stage of the fledgling, implying that sex-specific mortality is likely not the cause of sex ratio bias.

At ringing female offspring were by 11% heavier than males, confirming the reversed sexual size dimorphism that occurs in Eleonora's falcon. The size dimorphism was independent of rank, season or brood size, since female offspring were always larger than males. According to the literature, size dimorphism usually reflects sex-specific costs to the parents and hence, it can be the cause of many observed sex ratio biases (Komdeur and Pen, 2002). Body mass of females did not significantly vary with rank or season, but significantly varied with brood size. Although the pairwise comparisons showed no statistically significant interactions ($p = 0.06$) between female body mass and brood size ─an effect mainly caused by the accumulation of many outliers─ female body mass decreased with brood size. Males were significantly lighter in higher ranks and larger broods, whereas male body mass did not vary with season.

Sex ratio and year

Even though the annual bias in sex ratio was not statistically significant, more male offspring were produced during most years. This result is in agreement with the previous overall male bias that was observed in Eleonora's falcon (Ristow and Wink, 2004), as well as with the general theory that the sex ratio should be biased towards the less expensive sex, when daughters and sons are not equally costly to rear (Fisher, 1930). The opposite bias was observed in years when the number of offspring suddenly increased, but also when it suddenly decreased. These differences in the sex ratio bias might reflect changes in the external environment, such as an increase or decrease in food abundance (Benito and Gonzalez-Solis, 2007) or changes in the parental condition, such as the presence or absence of experienced and in good condition breeders (Morandini *et al.,* 2020; Ferrer and Bisson, 2003; Trivers and Willard, 1973). However, the lack of significance in those results, along with the missing information regarding food availability or parental condition, cannot lead to specific conclusions.

Sex ratio and hatching order (rank)

The observed female bias in the first rank and the male-biased higher ranks could support the 'intra – brood sharing – out hypothesis', according to which birds avoid brood reduction by producing the sex with the highest energetic requirements early in the brood (Carranza, 2004). In contrast, the 'intra – brood competitive equilibrium hypothesis' which suggests that a smaller sex is produced first within a brood to avoid the competition with its larger siblings, cannot be supported by the results of this study (Uller, 2006). Since female chicks of this species were heavier, mortality of the late-hatched females is more likely to occur than late-hatched males, given that the amount of food provided by the parents decreases with hatching order and assuming that females are generally more sensitive to harsh conditions than males (Torres and Drummond, 1997; Benito and González-Solís, 2007). In addition, body mass of females did not differ between ranks, whereas males of lower ranks were significantly heavier than those of higher ranks. This implies that high energetic requirements of Eleonora's falcon females are independent of the hatching rank, something that could explain why parents produce the most expensive sex first, when food supplies are adequate for the successful rearing of the female offspring.

A clear pattern of skewed first-rank sex ratios was previously observed in raptors, such as peregrine falcons (*Falco peregrinus*) (P. D. Olsen and Cockburn 1991) and bald eagles (*Haliaeetus leucocephalus*), where females are larger than males and within clutches the first eggs were predominantly females (Bortolotti, 1986b). Regarding the latter case, the author showed that the bias on the first rank was a strategy of the parents to minimize siblicide and maximize their fitness, as competition for resources between siblings resulted in an increased mortality of the last-hatched female chicks, indicating that females were more sensitive when were the last to be born within the brood. Similar sex ratio biases were observed in the European shag (*Phalacrocorax aristotelis*) where the larger sex was produced first. It was suggested that this pattern was the most efficient for the parents to avoid the increased competition between the larger and the smaller sex, subsequently the mortality of the larger sex when produced in higher ranks (Velando *et al*., 2002).

Sex ratio and hatching season (hatching date)

For all GLMM models, age was a better predictor of the sex than hatching date. This is a common effect caused by binning a continuous variable (age) into categories (hatching date), which might result in the loss of valuable information (Altman and Royston, 2006). In general, data dichotomisation might enhance the plotting of the data, but it can be problematic when it comes to regression (Altman and Royston, 2006). However, since hatching date derives from age, the interpretation of the results will be performed by using hatching date as the explanatory variable.

Seasonal variations in the sex ratio have been observed in many raptor species (Daan *et al.,* 1996; Smallwood and Smallwood, 1998; Olsen and Cockburn, 1991), as well as in Eleonora's falcon (Ristow & Wink 2004). The results of this study are in line with the existing literature and suggest that the early bias on females is a common strategy for many large raptor species (Komdeur *et al*. 1997). One possible reason for the observed early bias towards females, and the late bias towards males, could be the differences in age of first breeding between sexes (Newton, 1979). In Eleonora's falcon, females tend to breed earlier than males and female body size decreases with age, whereas the opposite pattern occurs for males (Ristow and Wink 2000). Thus, a few extra days might be more advantageous to females than to males if the probability of start breeding as yearling decreases with birth date for females (Komdeur *et al*., 1997).

The early bias on females could be, also, explained by Trivers & Willard (1973) parental condition hypothesis. In the case of Eleonora's falcon it has been observed that old and hence, experienced mates breed earlier, select the best breeding sites and are more efficient hunters and food suppliers (Wink *et al.,* 1993; Badami, 1998). For this reason, early breeders that are in better condition, and given that this condition will be inherited by their children, should produce more offspring of the costlier sex (Daan *et al.,*, 1996). In addition, since food availability is generally high early in the breeding season (Sheldon, 1998), according to the 'costly sex hypothesis' parents that breed early in season should produce more of the larger sex. Combining the aforementioned theories, it is suggested that females might benefit more than males by inheriting the good condition of their parents, and thus the sex allocation will lead to a female bias early in the season, and to a male bias as the season progresses. For example, in American kestrels (*Falco sparverius*) - where females are larger than males - it was proposed that larger females have an advantage in mating, whereas the size of males was found to be neutral without affecting their breeding success. This characteristic explained the reason behind more female offspring numbers for American kestrels when food supply was high (Olsen and Cockburn, 1991).

The results are in agreement with a previous study in Eleonora's falcon in which an early bias towards daughters was found (Ristow and Wink, 2004). The bias was explained by the increased fitness of the parents, and especially of males early in the breeding season. A similar seasonal bias towards females was reported in another large falcon species, such as peregrine falcon (*Falco peregrinus*) (Olsen and Cockburn, 1991), whereas the opposite patterns were observed in smaller falcons such as European kestrels (*Falco tinnunculus*) and lesser kestrels (*Falco naumanni*) (Komdeur and Pen, 2002). In the small falcon species, males that hatched early in the breeding season had a higher probability of breeding as yearlings, while in the peregrine falcon the authors suggested that heavy and healthy parents tended to produce more daughters (Navara, 2018; Olsen and Cockburn, 1991). Seasonal variation in the sex ratios are observed not only in raptors, but also in spotless starlings (*Sturnus unicolor*), European shags (*Phalacrocorax aristotelis*) and Linkoln's sparrows (*Melospiza lincolnii*) (Navara, 2018).

Sex ratio and orientation

The results indicated that the same sex ratio patterns were obtained both for sector and orientation. Due to the similarity between those two variables, orientation was a better predictor of sex than sector for the GLMM models, while the effect of orientation on the sex ratio bias is similar to that of sector.

In birds, the age of the breeders is known to be correlated with territory quality, with older breeders being more experienced, occupying the highest quality territories (Morandini *et al.*, 2020; Ferrer and Bisson 2003; Trivers and Willard, 1973). Since orientation can be an indicator of nest protection (Ristow and Wink, 1985), more experienced parents were expected to occupy the nests with the most favourable orientation and would produce more of the larger sex in this study (Trivers and Willard, 1973). More females were indeed produced in nests with eastern and south-southwest orientation, although. Although someone would expect the east – facing nests to be more exposed to the sun and heat, thus less suitable for breeders, the eastern site of the islet consists of more sheltered nests, under shade or in cavities and is greater exposed to the northeastern etesian winds that dominate during the post-laying season. The latter characteristic feature not only indicates a cooler and less humid microclimate for the rearing of the young, but also provides the parents with the advantage of enhanced foraging on passerines which migrate south (Xirouchakis *et al*., 2012). Similarly, more females were produced in the south-southwest facing nests, which are found in higher elevations, are more isolated and protected and provide a cool microclimate for the breeders. Contrarily, the nests which produced more males were those with a southern and western orientation, belonging to the driest and hottest sectors of the island, thus indicating a low-quality habitat for the rearing of the young, and subsequently the investment towards the smaller sex.

Sex ratio and brood size

The variation of the sex ratio in relation to brood size, with larger broods being female-biased, can be explained by the production of larger, female-biased broods early in the breeding season. According to the existing literature, parents overproduce the costlier sex when conditions are benign (Myers, 1978). Thus, it is suggested that fit parents of Eleonora's falcon can afford the investment in larger clutches, specifically in females, early in the breeding season when conditions are better and predominantly fit parents breed. The same pattern has been observed previously in Eleonora's falcon (Ristow and Wink, 2004), as well as in redwing blackbirds (*Agelaius phoeniceus*) (Myers, 1978) and in house wrens (*Troglodytes aedon*) (Bowers *et al.*, 2014).

Sex ratio and social environment

The 'Local Resource Competition hypothesis' could not be supported by the results of this study (Clark, 1978). If this hypothesis held true, in high quality territories or in increased food availability, a bias towards the less dispersive sex would be observed, namely towards males. In the current project, it was suggested that during benign conditions or in high quality territories, the sex ratio was biased towards females which are considered to be less philopatric than males (Ristow and Wink, 2000).

Adaptive significance of sex ratios

Evolutionary adaptations are evolved features of organisms that enhance their fitness in a particular environment and are shaped by natural selection (Burd, 2006). Regarding sex ratios, it is still debated whether the variety of results that demonstrate skewed sex ratios in response to environmental or social parameters, actually reflect functional adaptations (Navara, 2018) or are a consequence of maternal reproductive constraints (Alonso-alvarez, 2006). For example, the manipulation of the sex ratio might be the result of energetic constraints suffered by poorly nourished mothers, while attempting to produce more offspring of the larger sex. Such energetic constrains ,i.e. the increased energetic demands of large offspring in relation to the poor body condition of the mother, might limit the amount of lipids accumulated in the yolk of the egg which could lead to the production of other substances (i.e. hormones) that can alter the sex of the egg (Alonso-alvarez, 2006; Kappes *et al.*, 2015). Thus in this case, sex determination is not linked to an adaptation that aims to maximize the fitness of the parents but instead to a reproductive constraint.

For this reason, to test if sex allocation truly occurs in an adaptive manner, fitness should be measured. Fitness could be measured as the reproductive success of the sex of the offspring towards which the bias occurs (Komdeur and Pen, 2002) and by determining which sex will be likely more beneficial to the parents by helping in future reproductive attempts (Navara, 2018). Additionally, the gathering of maximum data regarding the life history of the species, as well as the monitoring of the external conditions that surround breeding pairs, is another important approach that aids the interpretation of the adaptive adjustments of animals' sex ratios (Navara, 2018). Lastly, multiyear studies are especially helpful for understanding the adaptive patterns of skewed sex ratios (Navara, 2018). Looking for consistency across years can be important at identifying strong drives of sex ratio variation.

Limitations and future recommendations

In the current study, the multiyear dataset, as well as the fact that similar results were obtained for all subsets of data that were analyzed, provided strong evidence of adaptive sex ratio biases in Eleonora's falcon. Nevertheless, there were some constraints that might have limited the interpretation of the results. For example, the lack of data for multiple colonies in Greece restricts the generalization that Eleonora's falcon adaptively biases the sex ratio of the offspring. Additionally, the missing information regarding food availability between seasons, sex – specific mortality, age of the parents or the absence of any measure of fitness do not allow for the accurate interpretation of the adaptive function of the observed biases.

For this reason, future work in Eleonora's falcon sex ratios should first examine multiple colonies of the Aegean Sea at the same time. In this case, the larger sample size and the variety of breeding habitats among the Aegean colonies might reveal even small deviations from sex ratio parity and it would reduce the likelihood of artefacts, allowing for more robust results that could prove the adaptive significance of the existing sex ratio bias (Tschumi *et al*., 2019). Furthermore, in order to avoid false interpretations of the observed sex ratio biases, it is suggested that additional measures of environmental and ecological parameters should be taken. Climatic data such as temperature and wind speed should be measured during the period of the study, in order to describe further the breeding conditions. Food abundance data of will, also, be useful for interpreting seasonal or yearly sex ratio variations that might be caused by an increase or lack of available food supplies, and a more detailed habitat assessment of the breeding colonies would be helpful in understanding sex ratio changes in relation to territory or nest quality. Another major parameter that future studies should take into account is sex-specific mortality and its relation with the offspring body condition. If it is proved that smaller females are more susceptible to death, and depending on the rank a female is born, the ‘intra – brood sharing – out hypothesis’ can be safely applied. Sex-specific mortality could be measured if DNA samples were obtained from the second visit on the field, followed by a third visit to record the remaining fledged individuals (Tschumi *et al*., 2019). Finally, parents’ body condition and age measures would be vital when it comes to applying Trivers & Willard (1973) parental quality hypothesis.

Despite the fact that including all of the aforementioned parameters might cost valuable time on the field, and in some cases, might be impossible to collect such data, it is suggested that the more variables are considered and are correlated with sex ratios biases, the more accurate conclusions regarding the adaptive significance of sex ratio skews would be drawn (Navara, 2018). Understanding sex ratios is not only important for revealing life history traits of wild animals, whose evolution depend upon complex trade – offs and interactions with the external environment, but also for applying the appropriate conservation measures in species that are affected by the current, rapid environmental changes (Wedekind, 2012). This study was the first thorough research of Eleonora’s falcon sex ratios in a colony with an excellent nest accessibility compared to the previous colony that was studied (Ristow and Wink, 2004). In conclusion, since Eleonora’s falcon is a species that is expected to be highly vulnerable in the case of a moderate climatic scenario (Dimalexis *et al.*, 2019) and there is currently little research going on in relation to the sex ratio patterns of its breeding colonies, it is proposed that more studies should be designed towards this direction and attempt to assess offspring sex ratio in a variety of highly accessible colonies across Greece and preferably on a multiyear basis.

5. Conclusions

In the current study, the sex ratio of Eleonora’s falcon varied both temporally and spatially, and consistency in the results across years was observed. Although, there are some restrictions in the interpretation of such results, it is proposed that the unequal costs of daughters and sons might play a major role in biasing the sex ratio of Eleonora’s falcon offspring. The quality of the habitat, the abundance of food, as well as the quality of the parents are probably the most significant drivers of sex allocation in this metapopulation, with all of them affecting positively the production of females. Therefore, it is indicated that the costlier parental investment towards females might lead to different sex allocation strategies for the parents to ensure the production of the most sensitive sex, namely females, when food provision is higher early in the breeding season or in lower ranks, and when fit parents can transmit their good condition to the sex that will be benefited the most. Given that the cheaper sex should be produced in higher frequencies in size dimorphic species, the observed tendency of producing females under benign conditions might be a species-specific strategy to counteract the natural bias towards males, and hence ensure the maintenance of a balanced offspring sex ratio.

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Appendix 1

DNA extraction protocol

(Miler *et al.*,1988 with some modifications)

1. Shake well to homogenize blood samples. Add 200 µl of blood into a new tube. Centrifuge at maximum speed for 15'(4^{o}C). Remove EtOH. Wash 2x (400 µl) with Wash buffer (10 mM Tris PH 8 / 100 mM EDTA)

2. Add 700 µl of a Lysis buffer (0.1M EDTA / 10mM Tris PH 8) to each tube.

3. Add 60 µl of 10% SDS to each tube.

4. Add 10 µl proteinase K (20 mg/ml) to each tube.

5. Put the tubes in a water bath at 55^{o}C for at least for 5 hours (or overnight).

6. Add 210 µl of a saturated solution of NaCl to each tube.

7. Mix thoroughly by shaking a few times (NOT vortex).

8. Centrifuge at maximum speed for 55 minutes (4^{o}C).

9. Transfer the supernatant into a new tube (2ml tube).

10. Add an equal volume (~900µl) of ice cold isopropanol to each new tube, close the tubes and mix by inverting a few times.

11. Leave the tubes at -20^{o}C for 2 hours minimum (or overnight).

12. Centrifuge at maximum speed for 45 minutes (4^{o}C) to pellet the DNA.

13. Immediately after the centrifugation, open the tubes and pour out the liquid.

14. Add (500 µl) 70% ethanol. Leave at 4^{o}C for 5-6 hours (or overnight).

15. Centrifuge at maximum speed for 45 minutes (4^{o}C), open the tubes and pour out the liquid again.

16. Leave the DNA to dry in the air 2-3 hours or at 37^{o}C for 1 hour.

17. Dilute the DNA in 100µl of Tris-HCl 5mM pH 8.5.

Appendix 2

Figures and Tables

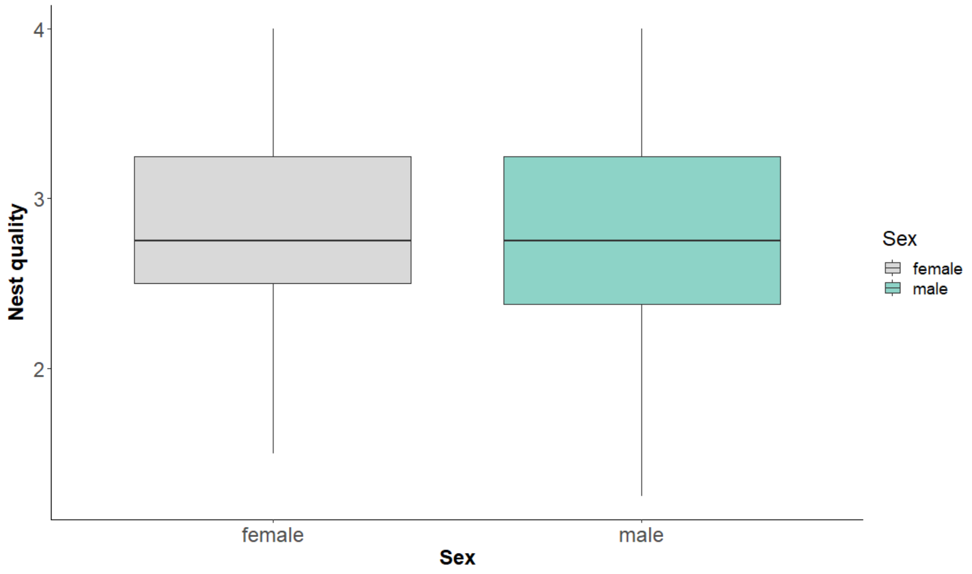

Figure 1. Distribution of nest quality between different sexes.

Table 1. Number and proportion of fledglings in relation to nest quality

Nest quality		**Rank 1**	**Rank 2**	**Rank 3**	**Total**
Mean ± SD	Females	2.83 ± 0.63	2.82 ± 0.59	2.82 ± 0.59	2.83 ± 0.61
	Males	2.74 ± 0.65	2.82 ± 0.68	2.81 ± 0.65	2.79 ± 0.66
Range	Females	1.5 - 4	1.5 - 4	1.5 - 3.75	1.5 - 4
	Males	1.25 - 4	1.25 - 4	1.25 - 4	1.25 - 4

Table 2. Number and proportion of fledglings in relation to nest type

Nest type	**Females**	**Males**	**Total**	**Sex ratio**
a	20	26	46	0.10
b	112	115	227	0.43
c	25	27	52	0.10
d	5	6	11	0.2
e	81	75	156	0.28
f	5	8	13	0.2
i	19	11	30	0.4
Total	**267**	**268**	**100**	**100**

*χ^2=4.05,df=6,**p=0.67,** no significant differences between nest types*

Table 3. Factors affecting the sex ratio – **Model 1** (With-losses analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	-2.436	1.172	-2.078	0.038 *
Type index	0.176	0.119	1.482	0.138
Sector index	0.067	0.098	0.688	0.491
Orientation	-0.002	0.001	-1.771	0.076
Rank	-0.342	0.132	-2.582	0.010 **
Age	0.103	0.036	2.882	0.004 **
Hatching date: Late	-0.246	0.296	-0.833	0.405
Hatching date: Early - Late	-0.124	0.330	-0.375	0.708
	Variance		**Standard Deviation**	
Year	0.034		0.184	

Table 4. Factors affecting the sex ratio – **Model 3** (With-losses analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	-2.441	0.730	-3.345	0.001 ***
Rank	-0.344	0.131	-2.619	0.009 **
Age	0.114	0.025	4.628	<.001 ***
	Variance		**Standard Deviation**	
Year	0.041		0.202	

Table 5. Factors affecting the sex ratio – **Model 4** (With-losses analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	-3.696	1.640	-2.254	0.024 *
Rank	0.374	0.838	0.447	0.655
Age	0.162	0.061	2.642	0.008 **
Rank : Age	-0.028	0.032	-0.866	0.387
	Variance		**Standard Deviation**	
Year	0.044		0.209	

Table 6. Factors affecting the sex ratio – **Model 5** (With-losses analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	-2.1467516	0.703627	-3.051	0.00228**
Orientation	-0.0019342	0.000938	-2.061	0.03928*
Rank	-0.3446597	0.133941	-2.573	0.01008*
Age	0.1160534	0.024269	4.782	1.74E-06***
	Variance		**Standard Deviation**	
Nest (Intercept)	2.70E-02		0.164	

Year	1.66E-08	0.0001

Table 7. Factors affecting the sex ratio – **Model 6** (With-losses analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	-2.1467301	0.703559	-3.051	0.00228**
Orientation	-0.0019342	0.000938	-2.062	0.03923
Rank	-0.3445999	0.134193	-2.568	0.01023
Age	0.1160493	0.024271	4.781	1.74E-06***
	Variance		**Standard Deviation**	
Nest	0.0181		0.1345	

Table 8. Sex ratio variation between years (Full-clutch analysis)

	Females	Males	Total	Sex ratio
2009	5	5	10	0.50
2010	6	12	18	0.67
2011	9	11	20	0.55
2012	3	7	10	0.70
2013	7	11	18	0.61
2014	16	17	33	0.52
2015	30	14	44	0.32
2016	21	16	37	0.43
2017	35	24	59	0.41
2018	8	13	21	0.62
2019	28	30	58	0.52
2020	16	18	34	0.53

Table 9. Sex ratio (proportion of males) in relation to rank and year (Full-clutch analysis)

	Sex ratio		
	Rank 1	**Rank 2**	**Rank 3**
2009	0.40	0.50	1.00
2010	0.63	0.57	1.00
2011	0.38	0.63	0.75
2012	0.75	0.50	1.00

2013	0.50	0.63	1.00
2014	0.31	0.77	0.43
2015	0.11	0.47	0.50
2016	0.33	0.47	0.57
2017	0.28	0.48	0.55
2018	0.56	0.67	0.67
2019	0.35	0.64	0.62
2020	0.46	0.54	0.63

Table 10. Sex ratio (proportion of males) in relation to hatching date and year (Full-clutch analysis)

	Sex ratio		
	Early	**Early - Late**	**Late**
2009	0.00	0.00	0.50
2010	0.17	1.00	0.91
2011	0.00	0.00	0.58
2012	0.33	1.00	0.80
2013	0.63	0.25	0.83
2014	0.44	0.00	0.75
2015	0.24	0.75	0.50
2016	0.27	0.67	0.88
2017	0.30	0.25	0.56
2018	0.56	0.00	0.73
2019	0.50	0.50	0.60
2020	0.48	0.75	0.56

Table 11. Factors affecting the sex ratio – **Model 1** (Full - clutch analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	1.465322	1.522505	0.962	0.33583
Type index	-0.098146	0.144766	-0.678	0.49780
Sector index	-0.046585	0.118198	-0.394	0.69349
Orientation	0.003637	0.001350	2.538	0.00705 **
Rank	0.176656	0.176656	-2.568	0.01114 *
Age	-0.094096	0.045489	-2.068	0.03859 *
Hatching	0.574655	0.380007	1.512	0.13048

date:late				
Hatching date:mean	0.365257	0.446252	0.819	0.41307
Clutch size	-0.079545	0.244763	-0.325	0.74519
	Variance	**Standard Deviation**	**AIC**	
Year	0.06905	0.2628	472.1	

Table 12. Factors affecting the sex ratio – **Model 3** (Full - clutch analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	2.490269	0.946179	2.632	0.00849 **
Orientation	0.003610	0.001174	0.00211 **	0.00211 **
Rank	0.435026	0.160473	2.711	0.00671 **
Age	-0.145613	0.032143	-4.530	<.001 ***
	Variance	**Standard Deviation**	**AIC**	
Year	0.119	0.3449	465.2	

Table 13. Sex ratio variation between years (Full-brood analysis)

	Females	Males	Total	Sex ratio
2009	10	7	17	0.41
2010	8	14	22	0.64
2011	12	15	27	0.56
2012	5	13	18	0.72
2013	13	19	32	0.59
2014	18	23	41	0.56
2015	33	27	60	0.45
2016	24	23	47	0.49
2017	39	32	71	0.45
2018	11	17	28	0.61

2019	30	32	62	0.52
2020	21	22	43	0.51

Table 14. Sex ratio (proportion of males) in relation to rank and year (Full-brood analysis)

	Sex ratio		
	Rank 1	**Rank 2**	**Rank 3**
2009	0.33	0.43	1.00
2010	0.60	0.56	1.00
2011	0.42	0.64	0.75
2012	0.70	0.67	1.00
2013	0.50	0.64	1.00
2014	0.39	0.81	0.43
2015	0.37	0.54	0.50
2016	0.43	0.53	0.57
2017	0.38	0.50	0.55
2018	0.50	0.73	0.67
2019	0.35	0.65	0.62
2020	0.42	0.56	0.63

Table 15. Sex ratio (proportion of males) in relation to hatching date and year (Full-brood analysis)

	Sex ratio		
	Early	**Early - Late**	**Late**
2009	0.00	0.00	0.44
2010	0.17	1.00	0.80
2011	0.00	0.00	0.58
2012	0.33	0.80	0.80
2013	0.56	0.43	0.86
2014	0.50	0.00	0.73
2015	0.32	0.83	0.69
2016	0.35	0.75	0.75
2017	0.39	0.33	0.55
2018	0.57	0.00	0.69
2019	0.51	0.50	0.54
2020	0.46	0.50	0.64

Table 16. Factors affecting the sex ratio – **Model 1** (Full - brood analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	2.629095	1.249256	2.105	0.03533 *
Type index	-0.216107	0.126502	-1.708	0.08758 .
Sector index	-0.069757	0.104185	-0.670	0.50315
Orientation	0.002436	0.001159	2.102	0.03551 *
Rank	0.557828	0.162766	3.427	0.00061 ***
Age	-0.078750	0.037680	-2.090	0.03662 *
Hatching date:late	0.333570	0.304907	1.094	0.27395
Hatching date:mean	0.164681	0.365043	0.451	0.65190
Brood size	-0.468291	0.188379	-2.486	0.01292 *
	Variance	**Standard Deviation**	**AIC**	
Year	0.004672	0.06835	614.9	

Table 17. Factors affecting the sex ratio – **Model 2** (Full - brood analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	3.528291	0.971535	3.632	0.000282 ***
Type index	-0.195398	0.125076	-1.562	0.118234
Sector index	-0.077561	0.104039	-0.74	0.455967
Orientation	0.002262	0.001148	1.970	0.048803 *
Rank	0.565870	0.162620	3.480	0.000502 ***

Age	-0.107123	0.028402	-3.772	0.000162 ***
Brood size	-0.479959	0.188098	-2.552	0.010722 *
	Variance	**Standard Deviation**	**AIC**	
Year	0.02084	0.1444	612.0	

Table 18. Factors affecting the sex ratio – **Model 3** (Full - brood analysis)

Variables	**Estimates**	**Standard Error**	**Z – value**	**P**
Intercept	3.252310	0.892648	3.643	0.000269 ***
Type index	-0.203620	0.124604	-1.634	0.102231
Orientation	0.002665	0.001014	2.629	0.008562 **
Rank	0.570388	0.162408	3.512	0.000445 ***
Age	-0.104859	0.028128	-3.728	0.000193 ***
Brood size	-0.499776	0.186084	-2.686	0.007237 **
	Variance	**Standard Deviation**	**AIC**	
Year	0.021	0.1449	610.6	