Why *K*not?

Exploration of Variation in Long-Distance Migration

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Why *Knot?*
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Voor Ans en Paul

De belangrijkste vormgevers van de omgeving waarin ik zélf opgegroeide

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Preface

When I was around 12 years old I used to help feed a flock of homing pigeons at their loft (among many other animals) after school time. One young pigeon somehow learned where I lived. At one day, it started showing up at my home to get some more food just after I had been at the pigeon loft. Because the distance between the loft and my house was at least one kilometre it took me about 10 minutes to get there. According to my parents, the pigeon always arrived home a few minutes ahead of me, with his arrival indicating my forthcoming return. Clearly, the pigeon did not follow me home, instead it must have found a more efficient route.

This is just one anecdote. In fact, I have been intrigued by the birdlife surrounding me for as long as I can remember. Their imminent presence in daily life sparked my interest for birds, not just for birds in general, but more specifically why they differ from individual to individual. Hidden lay a deeper question: what is I like to be a bird?
Key players in my scientific research are not pigeons, but red knots. However, my research emanates from the same question, yet more specific: what it is like to be a migratory bird? Through this research I got the opportunity to establish knots as individuals, rather than numbers, and explain that these differences hold some clues to the more general ecology of the species.

In my fascination for birds I clearly do not stand alone; in the Netherlands, public outreach websites with life streams broadcasting the developments from several nets box and their inhabitants (such as "*Beleef de Lente*"), attract one million unique viewers each spring. In addition, 80 000 households (this is 1 out of 100 Dutch households!) count and report their garden birds during the national bird counts organized by the Dutch Birdlife partner (i.e. *Vogelbescherming Nederland*) every winter. Interestingly, these popular programs are not

about rare or exotic species, rather, they represent common garden species such as jackdaws (*Coloeus monedula*) and great gits (*Parus major*). The fact that the makers choose to show hidden aspects of the life of species that live in our close proximity, and that we can easily relate to, may well be a factor explaining the success of these programs. For the simple reason that every tentative observer will soon recognize that a flock of birds does not consist of identical individuals. Instead, all birds show slight differences in appearance, have different personal preferences, show unique individual behaviour, and have complex social interactions.

From the joy I experienced as a child in observing and bonding with individual birds, I developed my scientific research into individual characteristics of red knots. This PhD came as an ultimate opportunity to submerge myself into the life of some red knots, building on all facts already known about this long distance migratory species. Above all, I got introduced to the scientific tools available to what I now know to be studies addressing consistent among-*individual differences* in animal species. Because I believe that the closer we are to understanding 'what it is like to be a bird (or any other animal) the more we are able, and willing, to appreciate and protect the animals and the habitats that are around us.

GENERAL INTRODUCTION

Eva M. A. Kok

Aim of the study

The aim of this thesis is to increase understanding of the development of individual migratory routines. I start with describing how satellite linked tracking devices enable us to look at the **structure** of individual migratory routines in much more detail than what was possible by means of tracking techniques that required manual superintendence. Where do migrants go when they leave the nonbreeding areas? What route do they follow? Now that previously invisible behaviour can be seen and analyzed, we can address a greater range of questions about migratory behaviour. In this thesis I also address possible **causes** of variation in migratory strategy. What are the developmental effects of individual differences in experience? What motivates individuals to embark on long distance migrations?

This introductory chapter starts off with a historical perspective on bird tracking studies and the migratory pathways they disclosed. Then I will discuss four key elements in bird migration studies: (1) circannual rhythms, (2) navigation and (3) orientation, and (4) development of migratory routines. Next, I will introduce red knots (*Calidris canutus*) and shortly review relevant research in 'knotology'. I subsequently build on the efforts of previous researchers to develop more detailed, and sometimes difficult and far-reaching, questions, which, I hope, will contribute to the body of knowledge on bird migration.

Migration

Historical perspective on bird tracking studies

Each year immense numbers of birds fly from their non-breeding grounds towards their breeding grounds, and back, some flying more than 10.000 km in one go, others making shorter flights and more stops along the way. These primarily north-south movements of birds cover all continents from west to east. This global phenomenon has captivated philosophers for centuries. These early day scientist were puzzled by the seasonal (dis-)appearance of birds. They asked questions like: Where do these birds come from in spring? Where do they go? How do they go there? And why do they take all this trouble in the first place?

One of the first documented attempts of a structured investigation into bird migration dates back to the Greek philosopher Aristotle, more than 2000 years ago. He formulated three hypotheses that could explain the seasonal re-occurrence of birds: hibernation, transmutation and migration. Aristotle assumed that in late summer the redstarts (*Phoenicurus phoenicurus*) transformed into robins (*Erithacus rubecula*) (see Figure 1) and that swallows hibernated at the bottoms of lakes. Alternatively, he hypothesized that the seasonal occurrence of birds could be explained by the birds moving south to avoid the cold of winter, and north to avoid the heat of summer (Aristotle 400 BC).
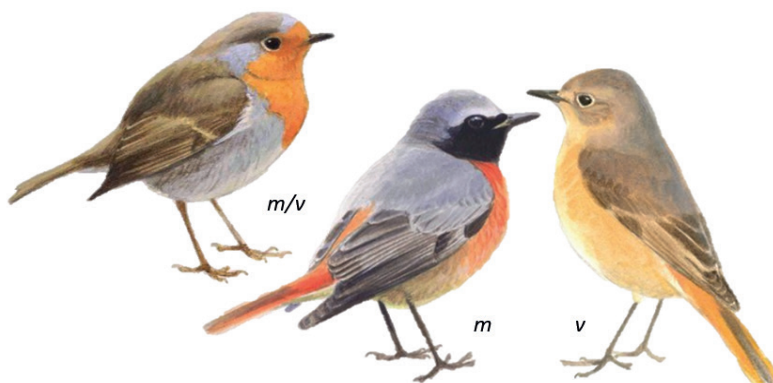
Figure 1. Aristotle's 'winter' robin and 'summer' redstarts. The close similarity in physical appears as well as behaviour between european robin (*Erithacus rubecula*) (12.5-14 cm) and common redstart (*Phoenicurus phoenicurus*) (13-14.5 cm) together with their complementing migratory appearance in Europe led Aristotle to believe that these birds biannually morph into one and the other. Image adjusted from www. vogelbescherming.nl.

Aristotle's ideas of hibernation and transmutation survived for almost 2000 years. Although these days we might think of these explanations as rather comical, every attentive observer in ancient Greece would have been able to notice the disappearance of redstarts, just before the robins arrived. The theory of hibernation, perhaps surprisingly, also came with elaborate reports that supported this theory. For example, reports of fishermen who would bring up hauls of sleeping birds (Magnus 1555). In a time that people were not as globally oriented as we are now, the practical and local explanations of transmutation and hibernation were probably more appealing to the human imagination than the possibility that birds were able to fly 1000s of kilometers over inhospitable terrains like oceans and deserts to other parts of the world. After all, these migratory movements primarily happened at high altitudes and were therefore invisible to the human observers on Earth.

The first anecdotal evidence for the migration hypothesis, and the idea that seasonally disappearing birds were in fact travelling between different continents, dates back to 1822 when a white stork (*Ciconia ciconia*) showed up in a German town, carrying along an African arrow protruding from its neck (Kinzelbach 2005). In the 18th century, studies related to bird migration started to resemble modern day migration studies. In 1757 Carl Linnaeus published *Migrationes Avium*, one of the first books on avian migration, in which he acknowledged that "*to resolve this intricate issue [of bird migration] it is essential that many people in various places on earth make careful observations and report them to the learned world*" (Linnaeus 1757). This principle of networks of observers still forms the basis for most current studies on the structure of migratory routines.

Circannual rhythms

In his Migrationes Avium (1757) Linnaeus describes how seasonality and related food availability may motivate birds to migrate. In order to cope with seasonally changing environmental conditions, as well as with different environmental conditions along the flyway, migratory birds show yearly reoccurring changes in physiology, behaviour and morphology according to a strictly organized schedule. These rhythms (usually referred to as circannual rhythms) are primarily driven by endogenous forces and subsequently adjusted by means of environmental factors such as seasonality and photoperiod (Gwinner 1986, 1996, 2003).

Migratory birds show seasonal changes in relevant morphological and labile physiological processes that are related to survival, endurance flight and reproductive behaviour (Gwinner 1996, Karagicheva et al. 2018). During the non-breeding season physiomorphic traits are primarily adapted to facilitate survival into the next breeding season. During the (pre-)breeding season migratory birds show physiomorphic changes that facilitate long distance flight and reproduction. Changes

include prenuptial molt of body feathers towards breeding plumage and simultaneous increases in fat and protein stores that function as fuel during flight or during the initial phase of breeding (Karagicheva et al. 2016, Lindström and Piersma 1993).

Additional preparation for successful migration and breeding include changes in preen wax composition (the chemical composition of preen waxes change during incubation, and fulfil different functions; Reneerkens et al. 2005) and in plasma corticosterone levels (a hormone involved in stress and immune responses; Landys-Ciannelli et al. 2002, Piersma and van Gils 2011) and enhanced cognitive performance (Rattenborg et al. 2004).

The increase of plasma corticosterone is also associated with increased levels of restless behaviour termed "Zugunruhe" (Kramer 1949). By means of so-called "Emlen" funnels (i.e. a blotting paper funnel with an ink pad base) a wide range of species was tested not only for their levels of activity (by means of the number of scratches made by the birds ink covered nails on the blotting paper) at the time that free living conspecifics start migration, but also for their preferred direction of movement (by means of the differences in density of the scratches in a certain direction; Emlen and Emlen 1966).

Navigation and orientation
The mechanisms of orientation that enable birds to follow migratory routes between stopovers include the (simultaneous) use of celestial- and magnetic compasses (Schmidt-Koenig 1960, Wiltschko and Wiltschko 1972, Emlen 1975, Kiepenheuer 1984). All too briefly, celestial orientation cues (such as the sun, stars and polarized light) can be used in relation to the position of the geographic poles (geographic north or south; Schmidt-Koenig 1960, Emlen 1975, Muheim 2006). The star compass is available during the night and guides night migrating birds towards the rotational center of the starry sky (Emlen

1975). During daylight hours, birds may orient by means of the sun, or sun related cues such as skylight polarization. The sun compass can be fixed, or related to an endogenous circadian time-compensation mechanisms to be able to correct for the predictable daily movement of the sun (Schmidt-Koenig 1960, Muheim et al. 2018). Skylight polarization patterns provide a geographic directional reference that can be used to calibrate other compass mechanisms (Muheim 2006). Because the geographic poles present a static (non-moving) goal, routes based on celestial compasses typically follow a geographic loxodrome (also called Rhumbline – a course with a constant heading towards a remote goal; Imboden and Imboden 1972). For a migrant to be able to travel along the shortest possible route on a globe (i.e. geographic orthodrome or great circle) would require constants adjustments of its course (Imboden and Imboden 1972).

Magnetic cues relate to inclination (i.e. dip) and/or declination (i.e. the deviation between geographic and magnetic north) of the Earth's magnetic field. A fixed magnetic compass is not sensitive to the polarity of the magnetic field lines but it uses the sign (i.e. positive or negative) of the inclination of the geomagnetic field to assess the direction away from the equator and towards the poles (Wiltschko and Wiltschko 1972, Kiepenheuer 1984, Wiltschko and Wiltschko 1996). The resulting route leads the bird along a constant magnetic course. By virtue of the constant heading the resulting route is referred to as magnetic loxodrome (Alerstam and Gudmundsson 1999b, Åkesson and Bianco 2016, Muheim et al. 2018). An inclination compass has been suggested to lead a migrating bird along a constant apparent angle of magnetic inclination (this is the angle of inclination of the Earth's magnetic field in relation to the migratory direction; Kiepenheuer 1984). Although theoretic courses along equal levels of magnetic inclination show close similarity to known migratory routes, to date empirical evidence for the use of such a compass mechanism is lacking.

Generally, the use of magnetic compasses is limited near the magnetic equator and poles and celestial cues become unreliable at high latitudes during summer when sunset is held off (Sandberg and Holmquist 1998). Given the varying strengths and limitations of each orientation mechanism migratory birds probably use a hierarchical assembly of different compass mechanisms that work simultaneously depending on the location and the time of the year and day (Able 1995, 1999, Muheim 2006). Rather than migrating between generally defined locations, recaptures of marked birds show that individuals are able to return year after year to the exact same breeding- and stopover locations at high precision (Gill 2019). The ability to return to previously visited locations, suggests that migrants are able to remember and navigate towards previously visited locations (Mettke-Hofmann and Gwinner 2003, Berbert and Fagan 2012).

Development of migratory routines

Many bird species show consistent among individual differences in various aspects of migration behaviour such as partner choice (Kentie et al. 2014), timing of enroute departures and breeding (Lourenço et al. 2011, Conklin et al. 2013, Gill et al. 2014, Carneiro et al. 2019, Verhoeven et al. 2019), (non-)breeding locations (Lok et al. 2011, Verhoeven et al. 2018, Méndez et al. 2020) and routes (Vardanis et al. 2011). At the population level consistent migratory routines may have evolved because birds that are consistent in their choices during migration have fitness benefits over individuals that explore a new strategy every year (Winger et al. 2018).

How individual differences in migratory routines come about is a question that is still unanswered. Age-related differences in spatial and temporal consistency in migratory strategy are indicative of an ontogenetic process (Thorup et al. 2007, Lok et al. 2011, Gill et al. 2014, Verhoeven et al. 2018, Dale et al. 2019, Oudman et al. 2020). In many bird species, individuals migrate south from the breeding grounds in

the year that they hatch, and do not migrate north to breed again until they are in their third year of life, after which they presumably migrate every year. In eurasian spoonbills (*Platalea leucorodia leucorodia*) it is known that they migrate south after hatching and that they only start breeding in their third summer. However, they only become fully faithful to the wintering location in their third winter. This is an indication that the development of individual migratory routines in spoonbills takes place in the first three years of life (Lok et al. 2011).

For species that migrate in mixed age flocks (such as whooping cranes (*Grus americana*), pigeons (*Colomba livia*), lesser spotted eagles (*Clanga pomarina*) and white storks (*Ciconia ciconia*) it is known that juveniles depend on experienced individuals during their first migration(s) to return along these learned paths in following years (Chernetsov et al. 2004, Mueller et al. 2013, Pettit et al. 2013, Meyburg et al. 2017). Thereby, juveniles on their first migration are prone to be affected by wind conditions and geography encountered while they adjust their flight path opportunistically in order to find high quality feeding grounds and avoid predation (Lindström et al. 2011, Vansteelant et al. 2017). The role of experience and learning in developing successful migratory routines is emphasized by the finding that birds become more efficient migrants with accumulating experience (Moore 1984, Thorup et al. 2007, Mueller et al. 2013, Rotics et al. 2017).

Red Knot

Red knots (*Calidris canutus*; hereafter knot, see Figure 2) are long distance migratory shorebirds that breed in the high arctic tundra and spend the remaining 9-10 months per year in intertidal areas around the globe. Based on small morphological differences and geographically separate breeding ranges, six subspecies of knots have been defined (Figure 3). Despite the fact that the genetic split of the subspecies is of

very recent origin (Buehler et al. 2006) and some subspecies co-occur on the same wintering-/staging site (such as in the Yellow- and Wadden Sea) the subspecies show very distinct patterns in circannual phenology and migratory trajectories (Buehler and Piersma 2008)

Knots show endogenously controlled circannual rhythms in phenotypic traits (such as preen wax composition, corticosterone levels, body mass, plumage and wing feather molt and digestive- and flight organ size) that are also maintained in captivity (Cadée et al. 1996, Piersma et al. 1999b, Piersma 2002, Reneerkens et al. 2007, Piersma and van Gils 2011, Karagicheva et al. 2016). During the non-breeding season knots weigh approximately 120 grams and have white breast plumage with grey coverts. Birds typically roam in large flocks at intertidal areas where they mainly forage on hard-shelled mollusks that they ingest whole and crush in their muscular stomach (called gizzard). Before departure on northward migration knots molt in to a rufousred breeding plumage speckled with black nuptial feathers and they increase in body mass to over 200 grams. Simultaneous body remodeling increases flight performance due to an increase in heart- and pectoral muscle (flight muscle) mass and a decrease in digestive organs mass and decreases in leg muscle mass and liver size (Piersma et al. 1993b, Piersma et al. 1999b, Morrison 2006).

breeding plumage

non-breeding plumage
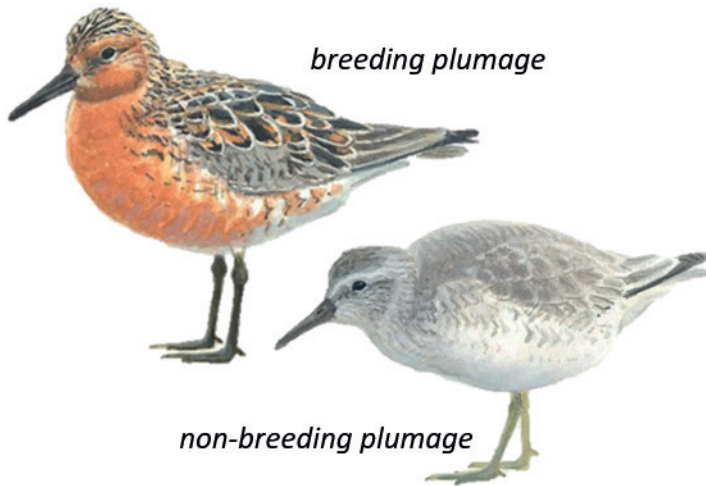
Figure 2. Red knot (*Calidris canutus*). The appearance of a mature red knot (23-25 cm) in breeding plumage (left) and non-breeding plumage (right). Males and females are indistinguishable in appearance. Images adjusted from www.vogelbescherming.nl.

Knots are able to rapidly adjust their digestive organ size depending on the seasonal variation in available resources and energetic demands (van Gils et al. 2005b). During the breeding season knots forage on soft-, invertebrate prey for which they do not require an extensive digestive system (Nettleship 1974). During the non-breeding season knots require much stronger and larger digestive machinery to be able crush the shells in their muscular gizzards. The profitability of different prey types varies depending on the relative flesh to shell ration (Zwarts and Blomert 1992). Individual birds are able to adjust their gizzard mass depending on the size and thickness of hard shell prey types in their diet that requires strong muscular gizzards while soft or thin shelled prey are relatively easier to digest. The development of a non-invasive method to measure gizzards (based on ultrasonography) facilitated studies on individual prey preference in relation to gizzard mass in living birds (Dietz et al. 1999a, Dekinga et al. 2001).

The availability of prey during the non-breeding season varies in space (Compton et al. 2013) and in time (Kraan et al. 2013) but also depends on the size of an individual's gizzard (van Gils et al. 2005a but see Mathot et al. 2017). Repeated measures of birds in captivity revealed that both gizzard mass and exploration behaviour (a proxy for habitat use) were repeatable (i.e. the variation in trait expression is higher among, than within individuals; Bijleveld et al. 2014, Mathot et al. 2017) and among-individual variation in gizzard mass correlates with space use in the wild. So, more explorative birds prefer high quality prey (i.e. high flesh to shell ratio) and have smaller gizzards than birds that are less explorative (Bijleveld et al. 2016, Oudman et al. 2016). In other words, birds that vary in exploration behaviour may select for different foraging conditions to which they physiologically adapt. However, how these consistent among-individual differences in behaviour come about is unclear.

For several decades now, our research group has been studying knots and their main prey species, gaining knowledge on their diet, habitat preferences, danger management, survival rates and annual routines in adults (Piersma 1994, van Gils 2004, Reneerkens 2007, Buehler 2008, van den Hout 2010, Leyrer 2011, Folmer 2012, Bijleveld 2015, Oudman 2017, de Fouw 2018). Compared to other species of (shore-) birds, multiple ecological attributes of the ecology of knots make this species especially suitable for fundamental ecological research. Because knots ingest their prey whole, intake rates can be calculated relatively easily (van Gils 2004) and the excretion of shell fragments allows for the identification of prey species in droppings (Dekinga and Piersma 1993). The development of ultrasonography to measure individual organ size allows for repeated measurements of digestive organs and related studies on prey preference (Dietz et al. 1999a, Dekinga et al. 2001). Compared to other shorebirds species such as sanderling (*Calidris alba*) and bar-tailed godwit (*Limosa laponica*), red knots are relatively easy to maintain in captivity because they seem

less prone to stress related responses when brought into (temporary) confinement. The establishment of the experimental shorebird facility at NIOZ, especially tailored to the needs of red knots, further allows for detailed measures under controlled conditions with knots in captivity. In terms of migratory programs, red knot migratory routines are well defined and relatively clear-cut because knots only make a few long distance flight that are interrupted by little, but distinct, stopovers *en route* (Dick et al. 1987, Piersma 1987, Piersma and Davidson 1992). However, due to the inaccessibility of the high latitudinal breeding grounds of the red knot, the ecology and development of juvenile red knots remains a major gap in our knowledge to date.

Two subspecies of knots (*C. c. islandica* and *C. c. canutus*), that show very distinct migratory routines but co-occur in the Dutch Wadden Sea during northward and southward migration, provide us with a unique system to explore the effect of environment and experience on the development of among-individual differences in phenotypic traits, as well as in migratory routines (Piersma 2007). With the recent development of small solar-powered satellite transmitters, time has come to start studying the developmental processes that contribute to known individual phenotypic variation in free-living knots from an early age onwards.
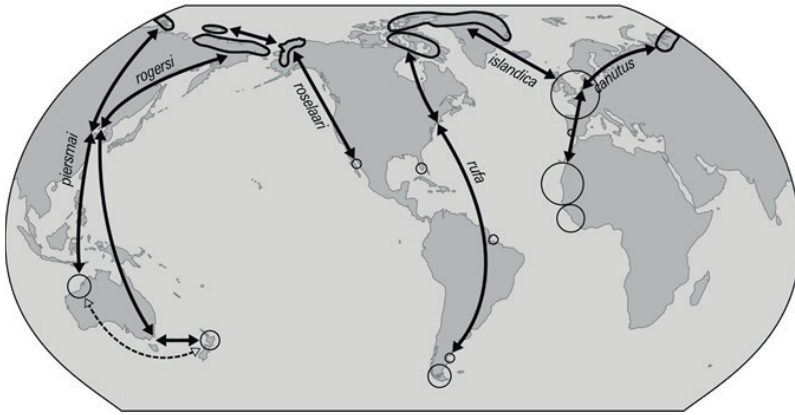
Figure 3. Migration routes. The six currently described subspecies of red knot (*Calidris canutus*) and their subspecies specific migration routes. Adjusted from Buehler and Piersma (2008).

This thesis

Scientific aim

Scientific research is founded on problems or mysteries and every research starts with a question. Depending on the kind of questions asked, one may learn about certain aspects of a phenomenon. Dating back to 400 BC, Aristotle argued that to increase one's knowledge about anything, one should seek information on four explanatory factors called the material-, formal-, efficient-, and final cause. Since then multiple prominent scientists have been inspired by Aristotle's original four cause and proposed a structural framework of questions to organize and increase (biological) knowledge (Mayr 1961, Tinbergen 1963, Laland et al. 2015, Hogan 2017). Admittedly these frameworks are rarely complete and categories are never defined sufficiently exclusively (Hogan 2017, see Table 1). Therefore any scientific conceptual framework should therefore be viewed 'philosophically' and primarily used to facilitate a pragmatic approach. Mayr (1961) suggested to divide all biological phenomena in *cause* and *effect*.

Tinbergen (1963) acknowledges Huxley for adding more complexity to studies in biology by formulating a systematic framework of problems including *causation, survival value* and *evolution* and he added a consolidating fourth problem; *development* (Tinbergen 1963).

For the structure of this thesis I have been motivated by Hogan's recent adaptation of Aristotle's fundamental framework for the specific use in studies on behaviour (Hogan 2017, see Table 1). What distinguishes Hogan's framework from the other frameworks is that he acknowledges that Aristotle's original causes are current problems (structure) as well as historical and future problems (development and evolution) that are relevant along different timelines. Consequently, Hogan's framework covers Aristotle's fundamental four causes while recognizing continuous developmental and evolutionary changes that historically gain a lot of traction in the field of behavioural ecology (Tinbergen 1963).

Traditionally, studies on bird migration focus on the structure of bird migration with a focus on describing current observations. 'What is the migrant doing?' The focal questions asked aim at describing assets of migratory behaviour such as the seasonal phenotypic adjustments, flight path, speed, altitude and duration. Answering these fundamental questions are especially valuable when the researcher's aim is to get more insight into the scope and variation in the expression of behaviour (Tinbergen 1963, Bateson and Laland 2013). 'Why questions' on the other hand usually take our understanding of any behaviour phenomenon a step further by inquiring into the causes and consequences of behaviour. A question such as "why do birds migrate?" can be answered in many different ways. For example, they migrate because (1) they learned to migrate while growing up (Mueller et al. 2013), (2) yearly light changes affect their internal circadian clock, which provides a stimulus for migration (Gwinner 1986, 1996); (3) moving away from diseases reduces maintenance costs by allowing

for reduced investment in the immune system (Piersma 1997), (4) it enables them to breed in places where chick survival may be higher because of greater food availability and fewer predators (Lindström et al. 2011). After close examination one can conclude that this single question provokes answers related to both the causes (1-2) and the consequences of migratory behaviour (3-4). These questions can further be divided in questions that refer to current migratory behaviour by individuals (2), to an evolved mechanism that has been selected for over multiple generations (3) or to individual developmental processes (1).

Thus far most behavioural work has been focused on the structure of current migratory behaviour together with the evolutionary fitness consequences of different strategies (What is the migrant doing, and how did this behaviour evolve?). At the same time questions related to the causes of current migratory behaviour (also referred to as mechanisms of control; Bateson and Laland 2013) and closely related questions about the development of migratory behaviour have largely been ignored (Bateson and Laland 2013, Beekman and Jordan 2017, Piersma 2018). However, specific answers to understanding behaviour may lay in non-genetic causes and in the developmental processes that continue throughout the life time of an individual (Stamps and Groothuis 2010, Laland et al. 2011). With the recent upswing of tracking studies evidence is accumulating that learning (a continuous cognitive developmental process) is important for the shaping of migratory routines (Meyburg et al. 2017, Vansteelant et al. 2017, Oudman et al. 2020).

Thesis outline
Migratory behaviour differs markedly between individual red knots. To get more insight in the breadth of individual variation present in the population the first part of this thesis revolves around traditional questions regarding the structure of red knot migration (see Table

1). The recent technological development of solar-powered PTTs light enough to be carried by red knots, provided us with the first opportunity to track red knots on a near-continuous basis and over multiple years. I start in **Chapter 2** with an analysis of the tracks obtained for a single individual of the *C. c. islandica* subspecies. This bird demonstrates the crossing of the inhospitable Greenland Icecap, and this enables us to reflect on the orientation capacities of red knots. Building on the tracking of many more individuals, in **Chapter 3**, we describe where red knots of the *C. c. piersmai* subspecies go during migration and confirm their suspected breeding grounds. In **Box 1** I summarize how we invested in individually tailored full body harnesses while exploring possible liabilities in the use of a full body harness in wild red knots.

In the second part of this thesis I go beyond the traditional questions and continue on some causes of variation in migratory behaviour with a special focus on developmental processes, including learning (see Table 1). Using cohorts of individuals of different age classes and collecting repeat observations on gizzard mass, pectoral muscle mass, and body mass of individuals over two years, we investigate how pectoral muscle mass responds to changes in gizzard mass (**Chapter 4**). This leads us to raise the question whether the individual trade-off between investments in gizzard and pectoral muscle mass found reflects the outcome of an evolved mechanism between fueling and flying.

In **Chapter 5**, we add scores of exploration behaviour measured simultaneously on the same individuals as in **Chapter 4**, and follow their development over time to investigate the ontogenetic causes of among-individual differences. I continue my investigation on the role of experience in the development of individual migratory routines in Box 2 where I present the preliminary results of the migratory behaviour of manipulated *C. c. canutus* knots originally caught as

juveniles and released after they have been experimentally 'imprinted' on the *C. c. islandica* route. In **Chapter 6**, we develop an experimental protocol to test if red knots are able to memorize and distinguish projected pictures of contrasting landscapes. Using this method we explore whether red knots show preferences for pictures of seasonally appropriate habitats, and if these preferences differ with age.

In the general discussion (**Chapter 7**) I will elaborate on how standardized behavioural assays can aid in studying the causes and consequences of behavioural variation. Because of the uncertainties about the co-variance between behaviours measured in highly standardized environments and the equivalent movements as free-ranging individuals, I suggest alternative methods to study the development of individual differences in migratory routines and the influence of experience. Finally, I discuss some of the implications of the findings in this thesis and make some suggestions for future research on individual differences in red knots.

Acknowledgments

Table 1. The scientific questions addressed in this thesis embedded in Hogan's structural framework for studies on behaviour. The first cause, the material cause or 'matter', is that what composes behaviour, i.e. the activity of the nervous system (Hogan 2017). Because the definition of 'matter' is the same regardless of the kind of behaviour expressed, this category is not further discussed in this thesis. The other three Aristotelean causes encompass the structure, cause and consequence of behaviour along different temporal axis (Current behaviour, Phylogeny and Ontogeny; Hogan 2017, see Piersma 2018 for an introduction on the use of this framework). Arguably the biggest question in the field of bird migration; 'Why do birds migrate?' can be placed in each of the nine sections below.

Aristotle's 4 Causes	Hogan	Current behaviour	Phylogeny	Ontogeny
Material Cause	Matter	X	X	X
Formal Cause	Structure	Ch2: What path do knots follow when crossing the Greenland Icecap? (and what does it tell us about orientation mechanisms used) Ch3: Where do *C. c. piersmai* migrate to, and where do they stop during migration?		
Efficient Cause	Cause	Ch6: Are knots motivated by seasonal changes in habitat preference?	Ch4: Why trade gizzard mass against pectoral muscle mass?	Ch5: How do age-related increases in gizzard mass and exploration arise?
Final Cause	Consequence	Box 2: How do individual migratory routines come about?		

CHAPTER 2

A RED KNOT AS A BLACK SWAN: HOW A SINGLE BIRD SHOWS NAVIGATIONAL ABILITIES DURING REPEAT CROSSINGS OF THE GREENLAND ICECAP

Eva M.A. Kok, T. Lee Tibbitts, David C. Douglas, Paul W. Howey, Anne Dekinga, Benjamin Gnep and Theunis Piersma

Despite the wealth of studies on seasonal movements of birds between southern nonbreeding locations and High Arctic breeding locations, the key mechanisms of navigation during these migrations remain elusive. A flight along the shortest possible route between pairs of points on a sphere ('orthodrome') requires a bird to be able to assess its current location in relation to its migration goal and to make continuous adjustment of heading to reach that goal. Alternatively, birds may navigate along a vector with a fixed orientation ('loxodrome') based on magnetic and/or celestial compass mechanisms. Compass navigation is considered especially challenging for summer migrations in Polar regions, as continuous daylight and complexity in the geomagnetic field may complicate the use of both celestial and magnetic compasses here. We examine the possible use of orientation mechanisms during migratory flights across the Greenland Icecap. Using a novel 2 g solar-powered satellite transmitter, we documented the flight paths travelled by a female red knot (*Calidris canutus islandica*) during two northward and two southward migrations. The geometry of the paths suggests that red knots can migrate across the Greenland Icecap along the shortest-, orthodrome-like, path instead of the previously suggested loxodrome path. This particular bird's ability to return to locations visited in a previous year, together with its sudden course changes (which would be appropriate responses to ambient wind fields), suggest a map sense that enables red knots to determine location, so that they can tailor their route depending on local conditions.

Introduction

In response to predictable changing environmental conditions, many birds seasonally migrate between two places on Earth, e.g. commuting from high latitude breeding grounds to lower latitude non-breeding grounds (Alerstam 1990a, Berthold et al. 2003, Winkler et al. 2016). The intermediate geographic routes followed by individuals are now being revealed by means of ever-smaller tracking devices (e.g. Gill et al. 2009, Klaassen et al. 2011). The main mechanisms of orientation (i.e. directed movement, Able 2000) enabling birds to navigate (i.e. charting a course to a remote destination) along migratory routes have been suggested to include the (simultaneous) use of celestial- and magnetic compasses (Emlen 1975, Able 1995, 1999, Wiltschko and Wiltschko 1996, Gould and Grant Gould 2012, Hansson and Åkesson 2014) in combination with other sources of information including landmarks (Alerstam 1976, Wiltschko and Wiltschko 2015), infrasound (Hagstrum et al. 2000), smell (Papi et al. 1971, Wikelski et al. 2015), and information communicated by peers (Chernetsov et al. 2004, Loonstra et al. 2019). The availability and function of most of these orientation mechanisms have been demonstrated in laboratory experiments (magnetic compass; Wiltschko and Wiltschko 1972, Sandberg et al. 1991, Sandberg 1992, celestial compass; Emlen 1975), and in experiments with free-flying birds with manipulated senses or endogenous clocks (Schmidt-Koenig 1960, Keeton 1971, Schmaljohann et al. 2013, Wikelski et al. 2015). How birds use, or combine, these orientation mechanisms during migratory navigation, remains largely unknown even today (Barrie 2019).

Travel along the shortest possible route between pairs of points on a sphere (the 'orthodromic pathway' or 'great circle route') requires continuous adjustment of the heading towards the goal (Imboden and Imboden 1972). For a moving individual, to be able to correct its course *en route* implies that the individual knows where it is relative

to its goal (i.e. have a map sense, Gould and Grant Gould 2012). An alternative strategy to navigate towards a remote goal is by vector navigation. Vector navigation enables migrating birds to maintain a pre-determined orientation (Able 1995, Guilford et al. 2011, Gould and Grant Gould 2012, Hansson and Åkesson 2014, Åkesson and Bianco 2016) and orient along geographic- and/or magnetic loxodrome routes (also called 'rhumblines'). These routes, by definition, are longer than orthodromes, but may be easier to achieve because of the constant heading (Imboden and Imboden 1972, Gudmundsson and Alerstam 1998).

To investigate the orientation mechanisms in operation during natural migration, theoretical flight path geometries based on assumptions of alternative vector navigation strategies can be compared with observed individual migration routes (Alerstam 1996, Sandberg and Holmquist 1998, Alerstam and Gudmundsson 1999b, Muheim et al. 2003, 2018, Guilford et al. 2011, Åkesson and Bianco 2017, Sokolovskis et al. 2018). The most informative comparison would be for summer migrations along trajectories close to the geographic and the magnetic poles, where the challenging 'cue environment' would help disclose possible orientation mechanisms. Here, constant daylight conditions complicate the calibration of time-compensated sun compass mechanisms while the stars are invisible and thus useless for orientation. At the same time, irregular geomagnetic field lines may complicate the use of magnetic cues close to the magnetic North Pole (Kiepenheuer 1984, Alerstam 1990a,b, Alerstam and Gudmundsson 1999b, Alerstam et al. 2001, Muheim et al. 2003, but see Sandberg et al. 1991, Sandberg et al. 1998, Åkesson et al. 2001).

We set out to investigate potential orientation mechanisms during the crossing of the Greenland Icecap by a red knot (*Calidris canutus islandica*). Of the six described subspecies of red knot, the *islandica* subspecies spends most of the year in the large intertidal systems of

northwest Europe. During northward migration most of the population visits known major stopover sites in western Iceland before they continue to migrate north to breed on the tundra in the Canadian High Arctic (Davidson and Wilson 1992, Piersma 2007, Buehler and Piersma 2008). *Islandica* knots have been suggested to cross the Greenland Icecap during both north- and southward migrations (Gibson 1922, Bent 1927, Morrison 1975, Dick et al. 1976). Observations of departing flocks in western Iceland were consistent with this suggestion (Alerstam et al. 1990, Gudmundsson 1993). Given that the destination area of this species is located in the challenging 'cue environment' close to the geographic- and the magnetic North Pole, no vector navigation strategy is known that would enable migrants to cross Greenland along a path with a constant geographic heading (Alerstam 1990b, Alerstam et al. 1990, Sandberg and Holmquist 1998). However, radar tracking on the coast of eastern Greenland suggested that red knots migrating between their spring staging area on Iceland and their breeding grounds on Ellesmere Island, Canada, travelled along a geographic loxodrome rather than the shorter orthodrome (Alerstam et al. 1986, 1990, Alerstam 1990b).

Acknowledging that unique and improbable observations can yield important insights in (ecological-) phenomena (the 'black swan' phenomenon, Taleb 2010), we present the details of four cross-Greenland flights by a single red knot to evaluate the possible orientation mechanisms used by this high latitudinal migrant. We compare the actual geometries of migratory tracks with the previously reported geographic loxodrome route (Alerstam et al. 1986), as well as the corresponding orthodromic routes. However, recognizing that the geometry of migratory tracks reflects the use of specific orientation mechanisms only in uniform worlds (Muheim et al. 2018), we also assess the influence of the moving medium, the winds, during the flights.

Material and methods

Paula, the focal bird

This red knot of the *islandica* subspecies was caught in mist nets at a high tide roost in the Dutch Wadden Sea (Richel, 53°17'31.67"N, 05°07'57.63"E) on 10 April 2016. The bird was an adult female, weighed 140 g, and showed moult of body feathers into the breeding plumage, indicating that she was physiologically preparing to breed. The bird, named 'Paula', received a metal ring (with inscription Z083055), a black flag and four colour rings (combination: N7PGRG, see Spaans et al. 2011).

Immediately after capture, Paula was moved to the Experimental Shorebird Facility of the NIOZ Royal Netherlands Institute for Sea Research on the island of Texel (53°00'N, 04°47'E), where she was housed with nine other red knots in an indoor aviary (4.5 m deep, 1.5 m wide and 2.3 m high). The aviary was equipped with rubber-coated concrete floors constantly irrigated with running seawater; it included a basin with sandy sediment from the Wadden Sea. The birds had free access to trout food pellets (Produits Trouw, Vervins, France) and natural prey (mud snails *Peringa ulvea*) as well as fresh water for drinking and bathing in a separate tray.

Paula was tagged with a pre-production prototype of a solar-powered rechargeable Argos 3 Platform Terminal Transmitter (PTT) weighing only 2.5 g (lxwxh = 20 mm x 10 mm x 7 mm + 210 mm antenna), produced by Microwave Telemetry, Inc. (2018). When sufficiently charged, this PTT transmits signals to the Argos satellite system every 50 s, and when signals are received by a polar-orbiting satellite, the perceived Doppler shift in signal frequency of successive transmissions is used to estimate the position of the transmitter (CLS 2016).

To allow for an acclimatization period in a safe environment away from predators and with ample availability of food, four days prior to release the PTT was mounted on Paula using a backpack configuration (Chan et al. 2016). Within minutes of being equipped with the PTT, Paula resumed normal behaviour and within a day, the PTT was completely preened under the back feathers and invisible except for the antenna. Before release, a few back feathers were clipped to better expose the solar panel; note that these feathers would have regained their original length after moult in autumn 2016. In an outdoor aviary we verified that the transmitter was functioning correctly. On 22 April, after 12 days in captivity and with a body mass of 151 g, Paula was released at mid-day during low tide in the Mokbaai on Texel (53°00'20.74"N, 04°45'48.60"E), together with five other red knots. (Paula is the knot most to the left in the picture on page 32)

Spatial analyses

Since we were especially interested in the trajectories of migratory flights, Argos data were filtered using a rate-based filter algorithm set for a high rate of speed (130 kph) and a relaxed minimum redundant distance (10 km) suitable for summarizing long-distance flights (Douglas et al. 2012). During filtering all standard-quality locations (i.e. location classes 3, 2, 1; for details on Argos location classes see CLS 2016) were retained while low-quality locations (i.e., location classes 0, A, B and Z) were retained only if they passed filter thresholds. Ground speed was calculated between successive retained locations by means of the great circle distance (function gdist() from the *Imap* package (Wallace 2012) and the time (UTC) elapsed between two locations.

Departure and arrival times and the intervening flights were determined from the visual inspection of the tracks (Google Earth ©). Departure locations were defined as the last location on land with a ground speed < 20 km/h (Chan et al. 2019b) and arrival locations as the first location on land with ground speed < 20 km/h. Intermediate locations were defined as 'migratory flights' and were labeled as

'northward' or 'southward' migration together with a unique identifier for each migratory flight by year (number of migratory flights per year is 4 for 2016, and 5 for 2017, see Figure 1 and Supplementary material Appendix 1, Table A1 for the migration scheme for both years).
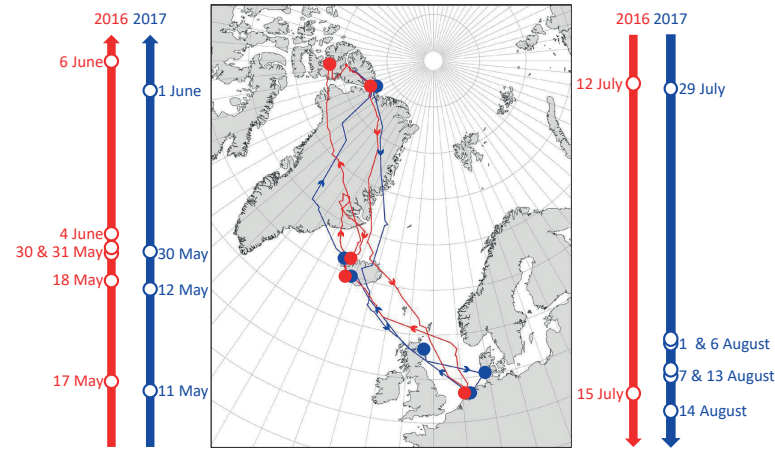
Figure 1. Flight paths of a single satellite-tracked red knot during 2 years.
Northward and southward migrations in 2016 (red) and 2017 (blue) are shown on an Azimuthal Equidistant map projection. Open circles in the timelines (left = northward migration, right = southward migration) correspond to solid circles in the map and represent stopovers in 2016 (red) and 2017 (blue). Arrowheads indicate the flight direction.

In contrast to previous models of PTTs (e.g. Gill et al. 2009, Senner et al. 2015, Chan et al. 2016), the 2 g PTT had no duty cycle, but rather transmitted whenever it was sufficiently charged. Under sunny conditions this provided numerous locations per day (average number of locations per day during migration 2016; n = 14 and 2017; n= 6). However, the transmitter was picked up more frequently at higher latitudes (since Argos satellites are polar-orbiting), and when Paula was in flight rather than on the ground. When the intermittent in-flight location data did not reveal appropriate departure or arrival times, we estimated those times by extrapolating the average speed (range 53 –

64 km/h) of each migratory flight from the last location in flight, or, for departure time, backward from the first location in flight. Throughout this manuscript we report measured distances based on filtered Argos location estimates. Since we did not continuously track the bird, local movements and slight deviations along flight paths may have been overlooked and thus overall distances moved may be underestimated. Hence, reported distances represent minimum distances traveled.

Orthodrome or loxodrome routes?

Geographic orthodrome and loxodrome paths between Iceland and Ellesmere Island were simulated by means of the functions gcIntermediate() and destPointRhumb() of the *geosphere* package (Hijmans 2019), with a resolution of 1 point per kilometer between previously assigned departure and arrival locations. Since Paula's tracks crossed Iceland without stops during both southward migrations (a pattern predicted by Dietz et al. 2010), we assigned 'pseudo-' arrival locations and arrival times by means of the locations where she first crossed the Icelandic coastline and the average groundspeeds of the respective migratory flights.

We calculated the mean distance and standard deviation of each transmitter location to their nearest locations on the simulated orthodrome and geographic loxodrome paths with the function dist2Line() of the *geosphere* package (Hijmans 2019). For each crossing of the Greenland Icecap, these deviations from the orthodrome and geographic loxodrome were compared by means of a two sided t test using the function t.test() of the *stats* package. All analyses were done in the R environment (v 3.6.3; R Core Development Team 2020).

Wind effects

To explore the effect of winds on migration path, we used NCEP Reanalysis data from http://www.esrl.noaa.gov/psd/ provided by the

NOAA/OAR/ESRL PSD, Boulder, Colorado, USA (Kalnay et al. 1996). We used the package *RNCEP* (Kemp et al. 2012a) to assess the wind data. The function RNCEP.interp() was used to obtain and interpolate u- and v-wind information in space and time for each received location (in degrees from north and in m/s; Kemp et al. 2012a). We did not measure flight altitude, but after inspection of the elevation profile of the Greenland Icecap (with main levels of altitude between 2000-2500 m with the highest peak at 3300 m above sea level (a.s.l.); see Alerstam et al. 1986), we obtained u- and v- wind components from the 700 mb atmospheric pressure level which corresponds to an altitude of roughly 3000 m a.s.l. (Alerstam et al. 1986). Flow assistance was then calculated for each location using the function NCEP.Tailwind(), assuming a fixed airspeed of 16 m/s (Shamoun-Baranes et al. 2010) and full compensation for wind drift (Chapman et al. 2011b, Kemp et al. 2012b).

To assess possible effects of winds on the geometry of the migratory paths during the flights over Greenland, we used the function NCEP. gather() (Kemp et al. 2012a) to obtain the prevailing wind speed and direction at the time of crossing. Given the temporal resolution (every 6 hour) and the spatial resolution (a 2.5° latitude x 2.5° longitude) of the available wind data (Reanalysis 2 dataset, Kanamitsu 2002), we queried the u- and v-wind components at 700 mb pressure level between 40°0'0''N, 90°0'0''W and 90°0'0''N, 30°0'0''E for the available time closest to the midpoint in time for each migratory flight.

To visualize general wind conditions and flow assistance in one map, and in the absence of information of the bird's flight altitudes, we used the 700 mb pressure level for wind calculations along the whole track. Following Shamoun-Baranes et al. (2010) and Alerstam et al. (1986), we repeated our analyses for the 500 and 925 mb pressure levels (5500m and 766 m a.s.l.). As we found that differences in flow assistance at different pressure levels were trivial, these results are not presented.

Results

In 2016 and 2017, the 2 g solar PTT on Paula successfully transmitted data during two northward (mid-May to early June) and two southward migratory journeys (mid-July to mid-August; Figure 1 and Supplementary material Appendix 1, Table A1 for more details). In 2016 Paula departed from the Wadden Sea on 17 May, i.e., 25 days after release, and flew nonstop to western Iceland, where she moved among multiple nearby staging sites, all known for red knots (Wilson 1981, Gudmundsson and Gardarsson 1992, Morrison and Wilson 1992, Wilson and Morrison 1992).

On 30 May 2016, Paula left Iceland for what turned out to be a round trip back to Iceland. She first flew northwest across the Denmark Strait (67°0'0''N, 24°0'0''W), then made a circuitous flight over part of the mid-eastern portion of the Greenland Icecap, before turning back to Iceland. This trip totalled 39 hours and 1859 km (Figure 1, Supplementary material Appendix 1 and 2, Table A1, Figure A1). Paula departed Iceland again four days later on 4 June 2016 and made a nonstop, 37-hour, 2404-km flight to Ellesmere Island, a known breeding area for *islandica* knots (Nettleship 1974, Whitfield et al. 1996, Figure 1, Supplementary material Appendix 1, Table A1). After some initial small-scale directional movements on Ellesmere Island between 8 to 26 June 2016, we began receiving highly clustered locations indicative of a bird tending a nest (95% of the locations were within 7.5 km of the period's average, consistent with Argos location errors expected to remain after filtering the raw data with a 10 km redundancy threshold). After 18 days, Paula resumed small scale directional movements towards the settlement of Alert (82°30'6"N 62°20'53"W), before departing on southward migration on 12 July in what was a single nonstop flight of 60 hours, covering 4091 km, to the Dutch Wadden Sea (Figure 1, Supplementary material Appendix 1, Table A1).

In 2017 Paula flew from the Wadden Sea to Iceland on 11 May, 6 days earlier than in the previous year, and staged for 17 days mostly at the same staging sites she visited in 2016. Still six days ahead of the 2016 northward flight schedule, she flew 41 hours over 2469 km to Ellesmere Island, this time arriving close to her departure location of the previous year, near Alert (Figure 1, Supplementary material Appendix 1, Table A1). Within three days, she moved approximately 250 km inland to a site that she also visited briefly after nesting in the previous year, and stayed at that locale for 26 days (95% of the locations were within 15.3 km of the period's average), indicating a breeding attempt. After subsequent small-scale movements, again in the direction of Alert, Paula flew back to Europe on 29 July, more than 2 weeks later than in 2016. In a nonstop flight of 68 hours she covered 3333 km to northern Scotland. After a stop of five days, she crossed the North Sea to the Wadden Sea near Sylt, Germany. Staying there for three more days, she finally arrived in the Dutch Wadden Sea on 14 August, one month later than in 2016. She thereby completed southward migration passage in 136 flight hours and covered 4422 km (Figure 1, Supplementary material Appendix 1, Table A1).

In 2016, Paula flew closer to the orthodrome than to the loxodrome path during both the northward (mean ± sd, orthodrome: 50 ± 36 km, loxodrome: 143 ± 101 km; t = -4.81, df = 37, p<0.0001) and the southward crossings of Greenland (orthodrome: 57 ± 44 km, loxodrome: 171 ± 108 km; t = -5.07, df = 35, p<0.0001; see Figure 2a,b and Supplementary material Appendix 1, Table A1). In 2017, during northward migration the locations deviated considerably from the two possible geometric paths (mean ± sd, orthodrome: 212 ± 192 km, loxodrome: 142 ± 136 km; t = 0.99, df = 18, p=0.33 see Figure 2c and Supplementary material Appendix 1, Table A1). During southward migration Paula flew closer to the orthodrome than the loxodrome (orthodrome: 66 ± 61 km, loxodrome; 115 ± 118; t = -2.59, df = 21, p=0.02; see Figure 2d and Supplementary material Appendix 1, Table A1).
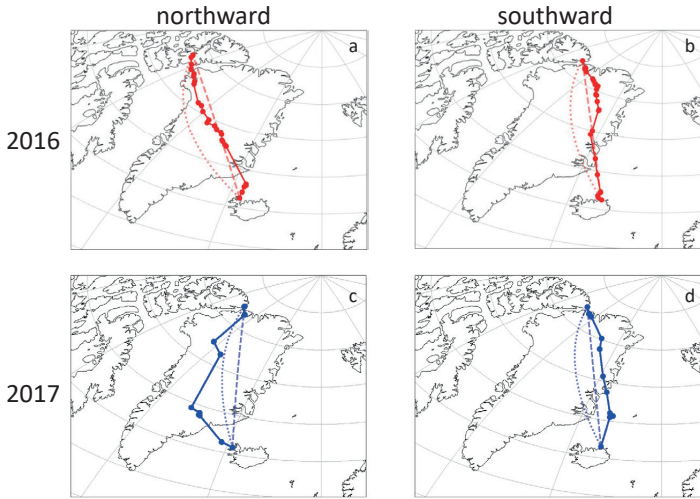
Figure 2. Orthodrome and geographic loxodrome paths. Maps show the northward (left) and southward (right) crossing of the Greenland Icecap in 2016 (red) and 2017 (blue) in relation to the orthodrome (dashed lines) and loxodrome (dotted lines) paths between Iceland and Ellesmere Island on an Azimuthal Equidistant projection. Red and blue circles show in-flight tracking locations.

In 2016, during the circuitous flight over the mid-eastern portion of the Greenland Icecap, Paula initially experienced positive flow assistance when she left Iceland. However, while she continued to fly north, she entered a narrow zone of headwinds (average flow assistance for this northbound flight = 0 m/s; Figure 3, panel 1a, Supplementary material Appendix 2, Figure A1). During the southbound return flight to Iceland, she also experienced headwinds (average flow assistance -5 m/s; Figure 3, panel 1 b).

During the subsequent complete northward crossing of the Icecap, Paula generally experienced low levels of flow assistance (average flow assistance = 1 m/s), with most wind support during the intermediate section. Before her arrival on Ellesmere Island she experienced weak headwinds (Figure 3c). At departure on southward migration Paula

experienced slight headwinds, however, favourable wind conditions and strong flow assistance prevailed during most of her crossing of the Greenland Icecap (average flow assistance = 3 m/s; Figure 3d). In 2017, Paula experienced weak to negative flow assistance during the northward flight over Denmark Strait and strong flow assistance during the second half of the flight across the Greenland Icecap (overall average flow assistance = 6 m/s; Figure 3e). Just as in 2016, in 2017 Paula experienced relatively strong flow assistance during southward migration when flying over the Greenland Icecap (average flow assistance = 4 m/s; Figure 3f).
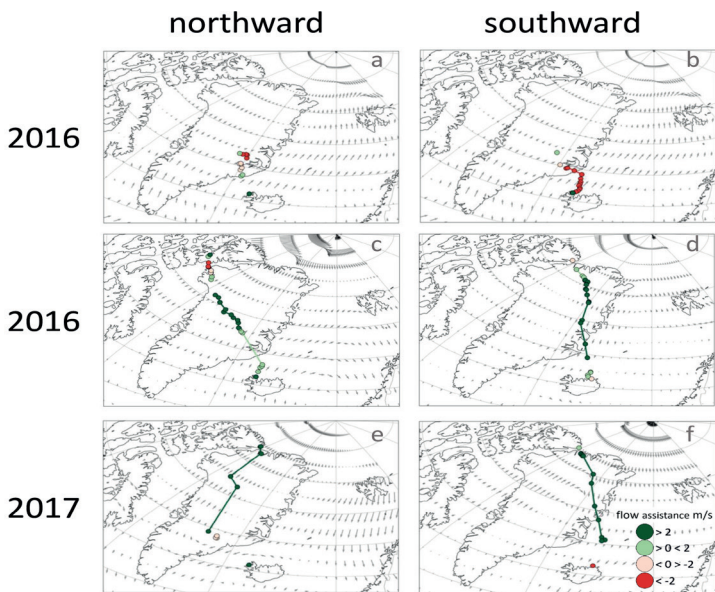
Figure 3. Flow assistance (m/s). Maps of calculated flow assistance at each tracking location during northward (left column) and southward (right column) migration, with positive flow assistance colored green (light green < 2 m/s, dark green > 2 m/s) and negative flow assistance (i.e. headwinds) colored red (light red > -2 m/s, dark red < -2 m/s. The spring 2016 circular flight (top row) that reached mid-eastern Greenland (a) before turning back to Iceland (b) , and completed migrations in 2016 (middle) and 2017 (bottom row) on an Azimuthal Equidistant projection. Gray arrows show wind speed and direction midway during the respective migration.

Discussion

Paula's flights confirm earlier predictions that red knots are able to traverse the Greenland Icecap during both north- and southward migration (Gibson 1922, Bent 1927, Morrison 1975, Dick et al. 1976, Gudmundsson 1993). However, contradicting earlier suggestions (Alerstam 1990b, Alerstam et al. 1990), she did not follow a geographic loxodrome when crossing the Greenland Icecap. In fact, in three out of the four crossings, the geometric shape of the track more closely resembled the shorter orthodrome route (Figure 2).

At high latitudes the use of a magnetic compass and a time-compensated sun compass could lead migrants along geographic paths that closely resemble orthodromes (Alerstam 1990b, Alerstam et al. 1990). The magnetic compass mechanism is based on the axial alignment of the geomagnetic field in relation to gravity (i.e. the geographic field) (Wiltschko and Wiltschko 1972, Kiepenheuer 1984). Since the magnetic- and the geographic poles do not coincide, birds orienting along a constant magnetic course (i.e. magnetic loxodrome) are led along a changing geographic course (which also varies in space and time due to the movement of magnetic north; Alerstam and Gudmundsson 1999b, Muheim et al. 2003, 2018). Flying along a constant magnetic course requires frequent recalibrations of the magnetic compass direction depending on the local magnetic declination (Alerstam and Gudmundsson 1999b, Muheim et al. 2003, Appendix 3). Migrants departing from Iceland could navigate along orthodrome like routes towards Ellesmere Island by means of orientation along a constant magnetic course towards the magnetic North Pole, which is currently situated close to Ellesmere Island (Alerstam et al. 1990, Supplementary material Appendix 3, Figure A2, A3). Orientation experiments at high latitudes suggest that songbirds are able to orient by means of geomagnetic cues even in close proximity to the magnetic North Pole (Sandberg et al. 1998, Åkesson et al. 2001).

In shorebird migrants with an endogenously controlled time keeping mechanism (Karagicheva et al. 2016), a time-compensated sun compass would enable the use of the daily movement of the sun to navigate along a geographic loxodrome (Muheim et al. 2018). However, during long distance migration across longitudes, maintaining a constant heading requires the recalibration of the internal circadian time-keeping mechanism (Schmidt-Koenig 1990). If recalibration is absent, at high latitudes each degree of longitude transected by a migrating bird will lead to a course shift of 1° in latitude (Alerstam et al. 1990, Alerstam and Pettersson 1991). Therefore, the use of a time-compensated sun compass without adjustment for longitudinal time shifts has been proposed to enable birds to fly along orthodrome-like routes (Alerstam et al. 1990, Alerstam and Gudmundsson 1999b, Muheim et al. 2018). The use of a sun compass calibrated in Iceland was previously suggested to enable orientation along an orthodrome route over the Greenland Icecap (Alerstam and Pettersson 1991, Alerstam et al. 2001, Gudmundsson et al. 2002), but empirical evidence is lacking. In both years Paula staged for two weeks on Iceland before crossing the Greenland Icecap. Her time spent at this stopover offered the opportunity to recalibrate her internal clock to match the local time on Iceland. This would have allowed her to orient along an orthodrome route over the Icecap by means of a sun compass.

Inconsistent with Paula's non-stop flights over Greenland are the many ring recoveries of knots along the west and east coast of Greenland that indicate that at least some individuals make intermediate stops (Alerstam et al. 1986; https://wadertales.wordpress. com/2019/08/15/sixty-years-of-wash-waders/). Stops along the Greenland coast were also observed in satellite-tracked brent geese (*Branta bernicla hrota*) and white-fronted geese (*Anser albifrons flavirostris*), waterbirds that migrate from east to west across much narrower parts of the Icecap in southern Greenland and thus avoid the highest mountain peaks (Gudmundsson et al. 1995, Fox et al. 2003).

Gudmundsson et al. (1995) suggest that the brent geese in their study followed a geographic loxodrome when crossing the Greenland Icecap by using topographical features in combination with a sun compass, under the assumption that the geese reset their endogenous clock from local Iceland time to Greenland time during a stop on the east coast of Greenland. Recent movement studies on smaller migrants carrying lightweight geolocators (i.e., northern wheatear *Oenanthe oenanthe leucorhoa*, Bairlein et al. 2012; purple sandpiper *Calidris maritima*, Summers et al. 2014; and ringed plover *Charadrius hiaticula psammodromus*, Léandri-Breton et al. 2019) suggest that most of these birds follow geographic loxodromes like the geese. However, some of the observed northward tracks suggest that individuals cross the Greenland Icecap above 65° N latitude, along routes that may approach orthodromes (Summers et al. 2014, Léandri-Breton et al. 2019). Unfortunately, the tracks end prematurely because the light-based geolocators used in these studies did not allow for estimation of locations under the constant daylight conditions present above the Arctic Circle during northern summer.

Winds across the Greenland Icecap are usually determined by a high pressure system northeast of Greenland and a low pressure system to its south (Alerstam et al. 1986, Liechti 2006). As a result, the prevailing winds across the Icecap come from the northwest, resulting in generally unfavourable winds during northward migration, but mostly favourable winds during southward migration (Alerstam et al. 1986). Confirming previous findings for shorebirds (Gudmundsson 1993, 1994, Alerstam and Gudmundsson 1999a, Green et al. 2004), Paula departed under (light) tail wind conditions from Iceland as well as from Ellesmere Island (Figure 3). She followed the orthodrome route most strictly immediately after departures and just before arrivals, drifting away from the shortest path at intermediate sections of her flights (Figure 2).

We considered the possibility that Paula's circuitous flight across part of Greenland in 2016 was an attempt to cross the Icecap, an attempt that was aborted because of deteriorating wind conditions during the traverse (Figure 3a,b, Supplementary material Appendix 2, Figure A1). A similar circuitous journey was actually observed in a satellite-tagged white-fronted goose by Fox et al. (2003). Although the goose departed from Iceland with following winds, it aborted the crossing of the Greenland Icecap and returned to the east coast of Greenland where it remained for the rest of the summer. In pectoral sandpipers (*Calidris melanotos*), circuitous flights initiated with following winds have been suggested to be the outcome of group behaviour when multiple individuals with different directional preferences act to lead the group along a compromised course until they split up (Krietsch et al. 2020).

In all four crossings of the Greenland Icecap, Paula experienced strong wind assistance at sections where she could afford to drift (i.e. at locations far from the goal locations). This was most pronounced during northward migration in 2017 when winds were considerably stronger than in 2016 (Figure 3e). While Paula departed from Iceland in 2017 in a westerly direction (rather than in a northwesterly direction to the breeding grounds), she turned almost 90 degrees north at a point where strong southerly winds supported her all the way to the breeding grounds (Figure 3e). Such distinct course changes have previously been explained by encounters with geographic features such as coasts, mountain ridges, rivers, and anthropogenic structures (Ottosson et al. 1990, Kuyt 1992, Gudmundsson 1993, Gudmundsson et al. 1995, Alerstam 1996, Alerstam and Gudmundsson 1999a, Åkesson 2007). Empirical studies show that, at least in homing pigeons *(Columba livia)*, visual guideposts are important when birds are close to their destination (Wilkie et al. 1989, Lechelt and Spetch 1997, Biro et al. 2007). The observation that Paula arrived on Ellesmere Island in 2017 at the location from where she departed from the breeding grounds in 2016, suggests that she targeted specific memorized locations (Kok

et al. 2020a). Orientation by means of visual landmarks during the crossing of the Greenland Icecap may be difficult if the landscape is homogenous. However, a broad overview of the landscape from the high altitude at which Paula may have been flying (Senner et al. 2018), together with the variable topography of the Icecap (reaching levels up to 3300 m a.s.l.; Alerstam et al. 1986), may contribute distinct visual cues to aid orientation.

As flying takes place in a medium that is moving itself at comparable speeds to the birds (Liechti 2006), migratory routes will only resemble the theoretically predicted geometric paths in a wind-still world, or when a bird completely compensates for wind drift (Alerstam 1979, Åkesson and Bianco 2016). Empirical evidence for the use of specific orientation mechanisms during natural migrations is therefore rather more challenging than what has been suggested (Gudmundsson and Alerstam 1998, Sandberg and Holmquist 1998, Åkesson and Bianco 2017, Muheim et al. 2018, Sokolovskis et al. 2018, but see Guilford et al. 2011); it requires careful strategic elimination of senses that rule out the use of 'all but one' orientation mechanism. Indeed, *'tracks only tell us where a bird was at a given time, but they do not tell us why and by what means it was navigating'* (Wiltschko and Wiltschko 2015). Nevertheless, the detailed observations of a single tracked individual (our 'black swan') crossing the Greenland Icecap on four occasions show us that red knots are in fact able to do just that. They may fly along the shortest, orthodrome-like path instead of the longer, previously suggested geographic loxodrome paths. Paula's ability to return to previously visited locations and fly primarily with following winds, together with the sudden, and fully appropriate course changes observed, suggest the use of a map sense that enables red knots to determine their location in relation to their goal and tailor their migratory route depending on previous experience and ambient wind fields.

Data accessibility

The data and reproducible code is available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.b8gtht795 (Kok et al. 2020c). The tracking data used in this study are also accessible on Movebank (movebank.org, study name "Red Knot Piersma Paula Greenland"

Authorship statement

E.M.A.K., A.D. and T.P. tested and set up the satellite transmitter. P.H. and T.L.T. developed and organized the registration of the transmitter and data collection. B.G. collected field data. E.M.A.K. analyzed the data with D.C.D and wrote the manuscript with T.P and T.L.T. All authors gave final approval for publication. Conflict of interest: PWH is the founder and CEO of Microwave Telemetry, the producer of the PTT used in this study. The authors declare no further conflict of interest.

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out under auspices of the Animal Experiment Committee (DEC) of the Dutch Royal Academy of Sciences (KNAW) (permit DEC-NIOZ 10.05). Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Electronic supplementary material

Appendix 1. Flight Itineraries

Table A1. Flight Itineraries for April 2016 - September 2017. nw= northward, sw=southward.

	2016	2017
Northward migration		
Total distance (km)	6404	4459
Total duration (h)	107	69
Number of migratory flights	3	2
nw departure from Wadden Sea	17 May	11 May
nw arrival in Iceland	18 May	12 May
nw departure round trip	30 May	-
nw arrival back on Iceland	31 May	-
nw departure from Iceland	4 June	30 May
nw arrival on Ellesmere Island	6 June	1 June
nw flight distance from Wadden Sea to Iceland (km)	2141	1990
Flight duration (h)	31	27
nw round trip (km)	1859	-
Fight duration (h)	39	-
nw crossing of the Greenland Icecap		
nw flight distance from Iceland to Ellesmere Island (km)	2404	2469
Flight duration (h)	37	41
length of orthodrome route (km)	2269	2093
mean deviation from location to orthodrome route (km)	50 +/- 36	212 +/- 192
length of loxodrome route (km)	2365	2129
Bearing (degrees from north)	305	329
mean deviation from location to loxodrome route (km)	143 +/-101	142 +/- 136
Conclusion	GC	NS
	t = -4.8057, df = 37.342, p-value = **2.519e-05**	t = 0.99243, df = 18.011, p-value = 0.3341

Table A1. Continued

	2016	2017
Breeding season		
start incubation	8 June	3 June
end incubation	June	>29 June
Southward migration		
Total distance (km)	4091	4422
Total duration (h)	60	68+39+29
Number of migratory flights	1	3
sw departure from Ellesmere Island	12 July	29 July
sw arrival Scotland	-	1 August
sw departure Scotland	-	6 August
sw arrival Sylt	-	7 August
sw departure Sylt	-	13 August
sw arrival in Wadden Sea	15 July	14 August
sw crossing of the Greenland Icecap		
sw flight distance from Ellesmere Island to Iceland (km)	2141	1990
Flight duration (h)	33	33
length of orthodrome route (km)	2134	2099
mean deviation from location to orthodrome route (km)	57 +/- 44	66+/-61
length of loxodrome route (km)	2190	2148
Bearing	143	145
mean deviation from location to loxodrome route (km)	171 +/- 108	155 +/-118
Conclusion	GC	GC
	t = -5.0729, df = 34.584, p-value = **1.325e-05**	t = -2.593, df = 20.978, p-value = **0.01698**

Appendix 2. Paula's circuitous flight over Greenland
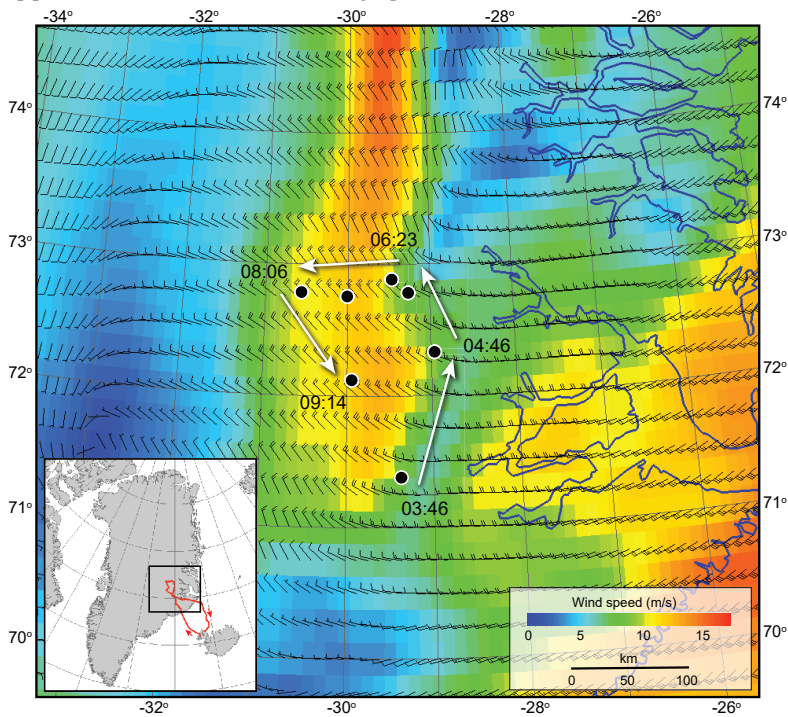
Figure A1. Paula's circuitous flight over Greenland during northward migration in 2016. Detailed wind conditions at the time when Paula made a circuitous flight over part of the mid-eastern portion of the Greenland Icecap, before turning back to Iceland. ERA5 wind speeds (700 mb) on May 31, 2016, at 06:00 UTC are color-shaded with wind-barbs denoting direction (Copernicus Climate Change Service, 2017, Unidata, 2020). Seven Argos tracking locations (black dots) obtained on May 31, 2016 are shown with generalized directional arrows and timestamps (UTC). After flying ~580 km from Iceland, a zone of headwinds that Paula encountered may have contributed to her decision not to undertake the remaining flight across the largely inhospitable Greenland Icecap to Ellesmere Island.

Appendix 3. Magnetic loxodrome

We obtained magnetic declination values at the departure locations and dates of the four migratory flights between Iceland and Ellesmere Island using the magneticField() function in the R package *oce* (Kelley and Richards 2019), which used the 12th generation International Geomagnetic Reference Field (IGRF, Thébault et al. 2015). For each migratory flight we adjusted Paula's initial geographic departure   bearing by the magnetic declination at the departure location to establish the magnetic bearing at departure. We used the destPointRhumb() function in the *geosphere* package (Hijmans 2019) with the magnetic bearing to advance Paula's migration in 10 km intervals, adjusting the bearing at each interval by the change in declination. We tuned Paula's initial departure compass bearings to establish magnetic loxodromes that led to Paula's observed destinations. The tuned departure compass bearings in 2016 and 2017 were, respectively, 328° and 345° for the northbound migrations, and 121° and 124° for the southbound migrations.

Figure A2. 2016 Magnetic loxodromes. Map of the northward and southward cross-ings of the Greenland Icecap in 2016 (solid lines) in relation to the orthodrome (dashed lines), the geographic loxodrome (dotted lines) and the magnetic loxodrome (solid grey lines) paths between Iceland and Ellesmere Island, shown in an Azimuthal Equidistant projection. The yellow asterisk shows the location of the magnetic North Pole in 2016 (194°N, and 86°E).

Figure A3. 2017 Magnetic loxodromes. Map of the northward and southward cross-ings of the Greenland Icecap in 2017 (solid lines) in relation to the orthodrome (dashed lines), the geographic loxodrome (dotted lines) and the magnetic loxodrome (solid grey lines) paths between Iceland and Ellesmere Island, shown in an Azimuthal Equidistant projection. The yellow asterisk shows the location of the magnetic North Pole in 2017 (187°N, 86°E).

BOX 1

A FOLLOW UP ON A TRANSMITTER ATTACHMENT METHOD FOR RED KNOTS

Eva M.A. Kok

Box 1

Since the development of individual, satellite linked tracking devices in the early 1990's the application of individual tracking devices has become increasingly popular in studies on bird migration (see Bridge et al. 2011 for a historical perspective). Satellite linked transmitters that operate on solar energy make it possible to track individuals over multiple years, or even during their whole life. Due to technological advancements, the lifetime of a transmitter increased tremendously and therefore existing attachment techniques such as taping and gluing (Raim 1978, Wilson et al. 1997, van Gils and Piersma 1999) no longer sufficed. This led to a search for more durable attachment methods (Rappole and Tipton 1991, Vandenabeele et al. 2013, Chan et al. 2016 and references there in).

The recent development of miniature satellite transmitters that operate on solar energy now allow for studies migratory routines in birds as small as red knots (Microwave Telemetry 2018). Instrumenting red knots with transmitters turned out to be particular challenging when compared with longer-legged wader species because red knots have no external 'knee' to facilitate for the loops of a the leg-loop harness to hold without damaging the bird (Chan et al. 2016). Tests and limitation to the use of two popular attachment configurations (leg-loop and back-pack style harnesses: sometimes also referred to as full-body harness or Chan-Piersma harness) are discussed elsewhere (Chan et al. 2016) but so far the use of leg-loop (Cohen et al. 2007) and the back-pack style harness on free flying red knots proved challenging (Chan et al. 2016, Scarpignato et al. 2016 but see Chapter 2 and 3, this thesis).

Building upon the harness developments by Chan et al. (2016) we continued to fine tune a backpack style harness for knots. We first tested the use of three different wire materials and individually tailored size configurations on 49 captive knots. Second, we summarize the most recent tracking attempts of wild red knots by attaching 2 g solar powered Platform Terminal Transmitters (PTTs) (Microwave

Telemetry Inc., Maryland, USA) attached to free flying knots with our preferred material.

Backpack development

In the period between 7 November 2017 and 26 June 2018 we attached 63 dummy transmitters by means of individually tailored backpack style harnesses on 49 knots (some individuals were fitted with another dummy after the first outfit failed). Dummy transmitters measured 20 mm x 10 mm x 7 mm (lxwxh) with a 210 mm antenna and weighed approximately 2 g. We compared three different materials for the backpack wires: 1) Tubular Teflon ribbon (2 x 0.5 mm). Teflon is the most commonly used material for transmitter attachments in tracking studies and it was previously successfully used for leg loop harnesses in multiple wader species (Shamoun-Baranes et al. 2012, Bom et al. 2014); 2) nylon coated stainless steel jewelry wire (0.6 mm diameter, Griffin, Germany); the use of this material was recommended by the producer of the 2 g PTT, Microwave Telemetry, for its smooth surface and increased transmission power. A very similar, but lighter nylon coated Nitilon wire (0.42 mm diameter) was used on a single knot that migrated successfully to and from the breeding grounds twice (Chapter 2, this thesis). However, when this lighter material was used for harnesses on red knots in northwest Australia and Mauritania transmission stopped within 4 months of deployment (northwest Australia 2016 and 2017 : n = 21, Chan et al. unpublished results; Mauritania 2017: n = 8, J. ten Horn pers. comm.). A few red knots in northwest Australia have been observed with broken harnesses after transmission failed, this is an indication that the material may have been too brittle; 3) Flyneema fishing line (1 mm diameter, de lijnenspecialist, Amsterdam). This is a lightweight, smoothly covered fishing line with a very strong Dyneema® core. A heavier type (2 mm) was recently successfully used for legloop harnesses on black tailed godwits (Loonstra et al. 2020).

Box 1

The neck and bodyloop in the Teflon and Flyneema harnesses were connected with a reef-knot fixed with super glue. The body loop was closed by means of two single brass crimp sleeves (10 mm). For the stainless steel wire backpack we used three double brass crimp sleeves to close the loops (see Figure 1).

General weekly health checks include the monitoring of circannual phenotypic rhythms in molt and fat storage. Following Chan et al. (2016) we added some backpack specific checks to assess the tightness of the backpack throughout the seasons (XS-XL), the abrasion of feathers under the transmitter as well as around the backpack wires, at the breast, under wings and in the neck (scores = 0-3).
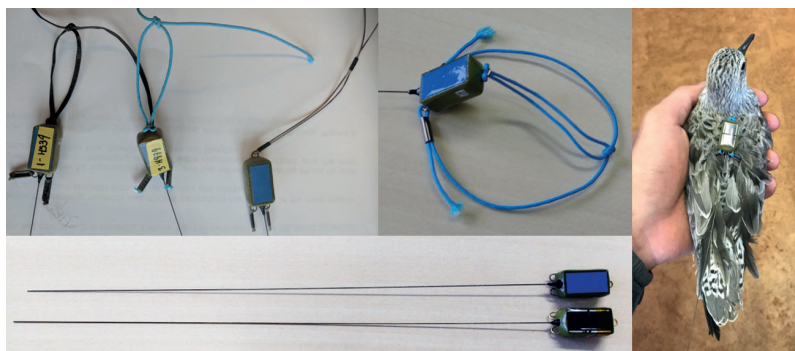
Figure 1. Harness development in captivity. Top left: Three different harness materials, Teflon, Flyneema, stainless steel (see text for specifications). Middle: Preferred backpack configuration with a 100 mm neck loop measured with calipers between the bolt on the transmitter and the breast knot. Bottom left: dummy (top), 2 g Argos PTT © Microwave Telemetry with solar panel (bottom). Right: red knot with PTT and Flyneema harness. Instructions to fit this backpack: mark the wire with different colors at 10mm intervals to standardize both size of the body loop (range 140-150mm). Close the body loop without fixing the two crimp sleeves. Place the neck loop over the head of the knot, adjust the wires and hold the transmitter in the preferred position. Make sure that the breast knot is in the right position and that the wires of the belly loop run down on the belly before going up under the wings. Pull all wing feathers through the body loop on both sides. Fix the crimp sleeves, and cut the surplus amount of wire.

Harness test

Our main conclusions from the harness tests confirm earlier findings. Similar to Chan et al. (2016) we found no evidence that harness attachment affects the natural seasonal changes in plumage and migratory fat reserves. Out of the 63 dummies applied, 30 were removed at the end of the experiment. The remaining 33 harnesses where removed prematurely due to excessive feather wear (n = 3), because the harness became too tight during migratory fatting (n = 6), because the knot got trapped with its bill in the harness (n = 18), or because of general health concerns (n = 6, see Table 1).

In line with Chan et al. (2016) we also observed that knots primarily get stuck with their bill in the wires within the first 24 hours after deployment (21 out of 63 cases). In approximately half of the attempts we have never observed a knot getting stuck in its harness (n = 34 out of 63 cases). Occasionally birds got stuck with their bill in the harness days, or even weeks, after attaching the harness (Figure 2) (14 out of 63 cases). On multiple occasions we have observed knots freeing themselves after their bills got stuck between the wires while preening. From these observations we conclude the initial entrapment observed in the first few hours after attaching the harness mainly results from a response that includes attempts at removal and enhanced preening efforts, behaviour that apparently faded with time in the majority of individuals. However, some individuals maintain the stress response for extended periods. One individual got trapped in its harness multiple times even after trying three different harness materials and sizes. In this case we saw no other option but to refrain from equipping this individual. The success of harnessing, clearly is individual.

Box 1

Table 1. Summary table containing 63 unique harness equipment attempts on 49 knots. The last column summarizes the removal reasons for each harness with 30 being removed at the end of the experiment, 18 because a knot got repeatedly stuck with its bill in the wires, 6 because the wires caused skin damage when the knot started accumulating fat when the migratory season started, 6 where removed because of other, unrelated, health concerns.

Harness material	number (n = 63)	neckloop (mm) Mean ±sd	body loop (mm) Mean ±sd	Total removed and why				
				Stuck	Tight	Wear	Other	End
Teflon	26	94±9	152±9	10	0	1	3	12
Stainless steel	19	98±10	164±8	4	5	2	2	6
Flyneema	18	89±7	149±8	4	1	0	1	12

Weekly physical examination of the knots revealed that initial feather shedding around the dummy and wires, which was predominantly occurring for the Teflon harnesses, ceased after the body plumage molt. Surprisingly the migratory body mass gain did not cause serious problems due to the almost 'fluid' texture of the fat reserves that gently accommodated the wires. In a few occasions the fine Stainless steel wires caused abrasion and bruising of the skin (Figure 3). Close inspection of the harnesses after removal revealed first signs of corrosion in the Stainless steel harnesses and crimps. The Teflon and Flyneema wires and single brass crimp sleeves were still in very good (new) condition at removal. When considering the feather and skin damage together with durability of the material and the ease of application, the Flyneema harness is our method of choice for the use in the field.

Box 1
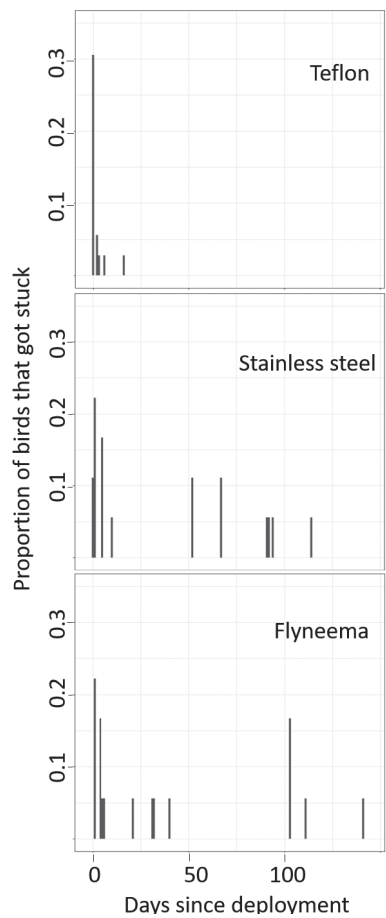
Figure 2. Bill-Backpack problems in captivity. The number of days that passed since deployment with the dummy before an individual got stuck with its bill in the wires of the harness. On the y-axis the proportion of birds of that cohort that got stuck on that particular day (x-axis). The dummy got removed in some occasions when a bird got repeatedly stuck. This can partly explain the peak < day 1, particularly visible in the upper 'Teflon' graph. When this happened a knot was fitted with a new backpack for which we started counting again at day 1. In 34 out of a total of 63 occasions we have never observed a knot being stuck in its harness.

Figure 3. Backpack problems in captivity. a) feather shedding in breast area, b) re-growth of feathers in breast area, c) accumulating fat under the skin, d) knot entangled in the wires of the backpack.

Field test

To date 20 knots have been mounted with 2 gram PTTs (© Microwave Telemetry) by means of a Flyneema backpack style harness. Two *C. c. canutus* knots were equipped with a transmitter in Parc National du Banc d'Arguin (Mauritania) in January 2018. Both transmitters successfully reported locations for 10 and 15 months after deployment. One transmitter eventually stopped transmitting during northward migration when crossing the Saharan desert. The knot has not been resighted and identified by means of its unique color ring combination since. The other transmitter was deployed on a knot that was previously held captive for a few weeks in January 2018 as part of a behavioural experiment. After release the transmitter was functional up to 15 months after deployment but in this time the knot never migrated. This individual was resighted multiple times at the non-breeding area in Banc d'Arguin at times that the transmitter was still functional (Table 2).

Of the 18 *C. c piersmai* knots equipped with a Flyneema backpack style harness in early 2018 in northwest Australia (NWA), we lost contact with ten transmitters before departure on northward migration. Four of these ten knots were later reported by means of color ring resightings in China and/or Australia, indicating that the losses resulted

Box 1

from transmitter and/or attachment failure rather than representing mortality. An additional three transmitters were reported from the north western Australian coast up to 2-10 weeks after deployment. These knots were never resighted. For the final three early failing transmitters (<48 hours after release) the knots have not been resighted since release. Based on this information, we cannot ascertain why we did not detect these knots after release; possibilities include depredation shortly after release (see Chan et al. 2016), transmitter related mortality (e.g., entanglement in harness), or transmitter failure. At least one transmitter performed poorly even during pilot tests. Of the remaining eight 'successfully' tracked red knots, one transmitter stopped sending locations during the first northward migration at the Chinese coast. An additional two transmitters stopped reporting, one after successfully reaching the breeding grounds, and another one during southward migration in Southeast Asia. None of these three knots have been resighted since. Two more transmitters successfully transmitted during one complete migratory cycle after which transmission stopped in the course of the non-breeding season in NWA. Both knots have been resighted since, indicating transmitter or backpack malfunctioning. The final three knots and transmitters were operational throughout two migratory cycles.

Because a transmitter and the backpack wires are usually completely covered by feathers the presence of a transmitter can only be confirmed in the field when an antenna is observed. Resightings of the 18 knots mounted with a Flyneema backpack in Australia in 2018 confirmed the presence of a transmitter for three transmitters that were still functional at the time. During eight observations no antenna was observed although two of those transmitters were still (poorly) functional at the time of observation (C. J. Hassell pers. comm.). Given the results of the harness tests in captivity we consider it highly unlikely that the harness broke. Together with deteriorating signal quality and quantity of the two transmitters on the knots for which no antenna

was recorded we wonder if damage and or breakage of the antenna may have led to transmission problems (the results of this tracking study can be found in Chapter 3 of this thesis).

Box 1

Table 2. Tracking in free living red knots. Dates in bold indicate that the bird has been resighted by means of individual identification of the color rings after the transmitter stopped reporting locations.

PTT ID	Capture location	Country	Acclimatization (h)	Capture day	Last resighted	Last tracking date	n days tracked
48959	Roebuck Bay	Australia	2	01/03/18	**26/04/19**	NA	0
48892[1]	Roebuck Bay	Australia	4	01/03/18	NONE	02/03/18	1
146242[2]	Roebuck Bay	Australia	2	01/03/18	NONE	02/03/18	1
48963	80 Miles Beach	Australia	24	16/02/18	NONE	19/02/18	3
48961	80 Miles Beach	Australia	24	16/02/18	**10/09/18**	26/02/18	10
48935	Roebuck Bay	Australia	2	01/03/18	**17/04/19**	14/03/18	13
48955	Roebuck Bay	Australia	2	01/03/18	**05/11/18**	17/03/18	16
48966	80 Miles Beach	Australia	24	16/02/18	**03/11/19**	13/03/18	25
48953	Roebuck Bay	Australia	3	01/03/18	NONE	08/05/18	68
48954	Roebuck Bay	Australia	2	01/03/18	08/04/18	13/05/18	73
168203	Roebuck Bay	Australia	3	01/03/18	NONE	23/10/18	236

Table 2. Continued.

PTT ID	Capture location	Country	Acclimatization (h)	Capture day	Last resighted	Last tracking date	n days tracked
48949	Roebuck Bay	Australia	1	01/03/18	**01/06/19**	11/06/18	250
48937	80 Miles Beach	Australia	24	16/02/18	**21/05/19**	16/11/18	273
48936	80 Miles Beach	Australia	24	16/02/18	NONE	05/12/18	292
48902[3]	Iwik	Mauritania	1	22/01/18	NONE	14/11/18	297
48895	Iwik	Mauritania	1	13/01/18	16/12/18	01/04/19	442
48951	80 Miles Beach	Australia	24	16/02/18	NONE	30/06/19	499
48968[4]	Roebuck Bay	Australia	2	01/03/18	13/04/18	04/11/19	613
48905	Roebuck Bay	Australia	4	01/03/18	27/04/19	28/11/19	637
48950	80 Miles Beach	Australia	24	16/02/18	20/08/19	21/12/19	673

1= Transmitter malfunctioned at first during testing, 2 = Harness fitting was redone when the bird got stuck with its bill during acclimatization period, 3= Although the transmitter kept reporting for months it did not move since 27/07/2018 when the signal abruptly stopped moving north after a period of continuous flight as part of what seemed liked northward migration, 4= Received locations from this transmitter suggest that it is not (anymore) reporting from the back of a (living) red knot.

Box 1

Conclusions

For long term tracking of red knots we recommend the use of a lightweight tracking device such as the 2 g PTT used here in combination with a Flyneema fishing line for the construction of a backpack style harness as developed by Chan et al. (2016). One of the main conclusions of the 2016 study by Chan and colleagues is that birds may have suffered increased predation risk due being equipped with a transmitter. We found no evidence for this. This could be due to the fact that the transmitters used here were lighter and contained no shield to protect the solar panel form feather coverage when compared to earlier studies (see Chan et al. 2016, Scarpignato et al. 2016).

From the captive experiments we concluded that a considerable percentage of knots equipped with a backpack design harness get entangled in the wires, and that this problem is most likely to occur within the first few hours after being fitted with the harness. Due to largely varying catching conditions and accessory quality in housing facilities the acclimatization period of the knots caught in NWA varied between 1 and 24 hours (Table 2). We found no correlation between the duration times that a knot was allowed to habituate to the transmitter and its subsequent free flying success. Of the group of eight knots for which we collected migratory behaviour, four were held captive for 1-4 hours, while the other four were kept for a day. The acclimatization period of the non-successful group followed a similar distribution (Table 2). Both findings are an indication that the problem of getting stuck in the harness is less relevant for wild knots. Maybe free flying knots are able to release themselves quite easily when they get stuck, or maybe captive knots preen excessively (e.g., due to stress), hence increasing the risk of getting stuck. Given the large individual variation in the ability to cope with the harness, we do recommend to observe freshly equipped birds closely before release and possibly refrain from tracking individuals that show signs of excessive stress.

Acknowledgments

3

WHEN A TYPICAL JUMPER SKIPS: ITINERARIES AND STAGING HABITATS USED BY RED KNOTS *CALIDRIS CANUTUS PIERSMAI* MIGRATING BETWEEN NORTHWEST AUSTRALIA AND THE NEW SIBERIAN ISLANDS

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The ecological reasons for variation in migration strategies, with some populations migrating across thousands of kilometres with one or few refuelling stops, while others stop much more often, remain to be pinned down. To contribute to illuminating these issues, we examined the seasonal migration of individuals of one subspecies (*piersmai*) in a shorebird well known for its long-haul migrations (red knot *Calidris canutus*). We tagged 18 red knots on the nonbreeding grounds in northwest Australia with 2 g solar-powered satellite transmitters (PTTs) and in two years we obtained detailed information on eight return trips to the Arctic breeding grounds in eight individuals (with evidence for two similar return migration in two other birds). We confirm that *piersmai*-knots from NW Australia breed on the New Siberian Islands and that they stage along the coasts of eastern Asia, especially China. In four cases, red knots arrived on the tundra on 8-12 June, in two cases only in late June. Southward departures mainly occurred in the last week of July and the first of August. Despite nine non-stop flights of over ca. 5000 km (with a maximum of 7100 km) in six individuals, quite unlike what has been described for other subspecies, the red knots made several stops of up to a week especially during northward migration - rather than staging at a single location for multiple weeks. In this process they 'hugged' the coast of China and made detours of up to 1500 km compared to direct, great circle route, migrations. Along the coast of China the stopping sites were characterized by an abundance of the bivalve *Potamocorbula laevis*, previously identified as superb food. We discuss the surprising discovery of the use of multiple food-rich staging sites in a subspecies of red knot in decline because of elevated mortality away from the northwestern Australian 'wintering' grounds.

Introduction

There are probably good biological reasons for some birds to breed in one part of our planet and spend the rest of the year in another; the published search for these reasons now occupies a few meters of bookshelf, but the field was ably summarized by Newton (2008). As just one example, the long-distance migrating shorebirds breeding in the Arctic combine their reproductive activities on the tundra with a life on soft-sediment seashores, flying as far south as the Sub-Antarctic for survival (see generalizations in Piersma 1997, 2003, van de Kam et al. 2004). Such coastal shorebirds show a range of food specializations and among them the red knots *Calidris canutus* combine visual hunting for surface-living arthropods on the tundra (Martin and Piersma 2009), with probing for hard-shelled prey (usually bivalves) on intertidal sedimentary flats (e.g. van Gils et al. 2006, Quaintenne et al. 2010). Red knots possess a unique sensory system for the remote detection of hard objects in wet soft sediments (Piersma et al. 2009, de Fouw et al. 2016). Away from the tundra breeding grounds, they are highly social and often occur in large flocks (Piersma et al. 1993, Bijleveld et al. 2016, Oudman et al. 2018), which is also part of a strategy to avoid depredation by falcons (van den Hout et al. 2010). As suitable habitats are rare and widely dispersed across the globe (e.g. van Gils et al. 2005b), the long migration flights of red knots (Piersma and Davidson 1992, Piersma et al. 2006, Shamoun-Baranes et al. 2010) may be considered a consequence of their ecological specialization.

Despite the great knowledge on the connectivity of five red knot populations/subspecies worldwide (Piersma and Davidson 1992, Tomkovich 1992), as recently as 2001 P.S. Tomkovich described a sixth subspecies, *C. c. piersmai*, on the basis of museum skins of breeders collected on the New Siberian Islands (Tomkovich 2001). A single resighting in NW Australia on five occasions between November 1995 and September 1996 of a red knot that was individually colour-marked

on 10 July 1994 at the Faddeyevski Island, New Siberian Islands group, Russia (Lindström et al. 1999), as well as biometric data and plumage observations (e.g. Verhoeven et al. 2017), were all consistent with the idea that many *piersmai* birds spend the northern winter in NW Australia — even though some migrate as far as New Zealand (Tomkovich and Riegen 2000).

The observation that red knots departed northwards from NW Australia late into May, led Battley et al. (2005) to predict: (1) the use of high quality shellfish food at suggested staging areas along the Yellow Sea, (2) and arrivals on the New Siberian Island breeding grounds in early June. The prediction of high food quality and abundance in the Yellow Sea was confirmed by Yang et al. (2013, 2016) for red knots staging in Luannan County, Bohai Sea (see also Rogers et al. 2010, Yang et al. 2011, Hua et al. 2013, and see yearly field reports at http://globalflywaynetwork.com.au/bohai-bay/reports-and-papers/).

A sophisticated quantitative analysis has shown that large numbers of red knots stage at this site for 5-9 days only (Lok et al. 2019), implying that there must be several other staging sites *en route*. Does this mean that, in contrast to what red knots have been shown to do elsewhere in the world (Piersma et al. 2005, Piersma 2007), in the East Asian-Australasian Flyway they do not necessarily show 'long-jumping' (Piersma 1987)? If not, where are the additional staging areas? Can staging areas be characterized in particular ways in terms of food availability? Obviously, it should also be established that *piersmai* red knots really breed in the New Siberian Islands.

In 2018 the successful miniaturization of satellite tags by Microwave Telemetry helped us to obtain a good number of tracks of red knots captured in NW Australia in 2018. From 2017, we also surveyed a good number of potential stopping sites for birds and macrobenthic bird food along the coast of China (Chan et al. 2019a,b, Peng et al. 2020). In

the present contribution we provide an analysis of the temporal and spatial characteristics of the seasonal migrations of red knots captured in NW Australia, we assess the lengths of their non-stop flights and the number of stopping sites used, and examine the possible food resources at coastal habitats used as stopping sites during northward and southward migration. With the small bivalve *Potamocorbula laevis* as the main known food of red knots at a key staging area in China (Yang et al. 2013), we focused on comparing densities of the small bivalve *Potamocorbula laevis* between visited and non-visited sites along the Chinese coast.

Material and methods

This study is part of an international collaborative effort by Global Flyway Network and associated institutions to study the demography and migration ecology of several representative shorebird species along the East Asian-Australasian Flyway (e.g. Piersma et al. 2016, Lok et al. 2019, Chan et al. 2019b). In February-March 2018, using cannon nets, we captured red knots at Roebuck Bay and Eighty-Mile Beach (19.34°S, 121.41°E). After capture, birds were measured and weighed, and a small blood sample was taken for molecular sexing (see van der Velde et al. 2017 for protocol). Birds were aged based on plumage characteristics (Higgins and Davies 1996) and adults were selected for satellite tagging. We tagged 18 red knots with solar-powered rechargeable Argos 3 Platform Terminal Transmitters (PTT) weighing only 2.5 g (lxwxh = 20 mm x 10 mm x 7 mm + 210 mm antenna), produced by Microwave Telemetry, Inc. Transmitters were deployed using a body harness (Chan et al. 2016) made of 1 mm thick Flyneema (de Lijnenspecialist, Amsterdam, The Netherlands). The birds were kept indoors and observed for a few hours (Roebuck Bay) up to 48 hours (Eighty-Mile Beach) to ensure acclimatization to the transmitter and harness. We then released the birds at the capture location. The tagging work was carried out under Regulation 17 permits SF 010074,

SF010547 and 01-000057-2 issued by the West Australian Department of Biodiversity, Conservation and Attractions.

When sufficiently charged, the PTTs transmit signals to the Argos satellite constellation every 50 s (i.e. there were no duty cycles), and when signals are received by a satellite, the perceived Doppler shift in signal frequency of successive transmissions is used to estimate the position of the transmitter (CLS 2016). Argos data was filtered using a rate-based filter algorithm set for a high rate of speed (130 kph) and a relaxed minimum redundant distance (10 km) suitable for summarizing long-distance flights (Douglas et al 2012). During filtering all standard-quality locations (i.e. location classes 3, 2, 1; for details on Argos location classes see CLS 2016) were retained while low-quality locations (i.e., location classes A, 0, B, and Z) were retained only if they passed filter thresholds.

In a first exploration to identify potential stopovers each location recorded during the migratory season was identified as 'flying' when 3 consecutive locations had a groundspeed of at least 10 km/h. When a birds' ground speed did not exceed 10 km/h for at least 24h this period was marked as stopover. Departure and arrival times and position at stopping sites and intermediate flight bouts were verified by visual inspection of the filtered tracks (Google Earth ©). Departure locations were roughly defined as the last location on land with a groundspeed < 20 km/h (Chan et al. 2019b). Arrival locations were defined as the first location on land with groundspeed < 20 km/h. To assess the amount of extra flight kilometers when compared to the shortest path great circle distances were calculated between arrival and departure locations by means of the distHaversine() function from the *geosphere* package (Hijmans 2019). Since we did not track continuously, reported distances represent minimum distances traveled while taking the remaining location errors of the Doppler derived locations into account. We have not accounted for the logic that with more high-quality locations, the

reconstructed track become longer.

From early April to late May 2018, we sampled the benthos at 21 intertidal flats along the coastline of China, extending from Dongliaodao in the far south to Panjin in the far north (see Peng et al. 2020). At each site we sampled macrozoobenthos across grids (for the rationale, see Bijleveld et al. 2012). Depending on the local situation, gridlines were set either 50 m or 500 m apart to cover the area from the coast to the low water line. Across the 21 sampling sites, a total of 838 sampling stations were visited. At each station a sediment core with a surface area of 0.019 m^2 was taken to a depth of 20 cm and washed over a 0.5 mm sieve. The sieved samples were stored frozen before analysis in the laboratory where shellfish were counted and identified to species level using a dissecting microscope. For further details, see Peng et al. (2020).
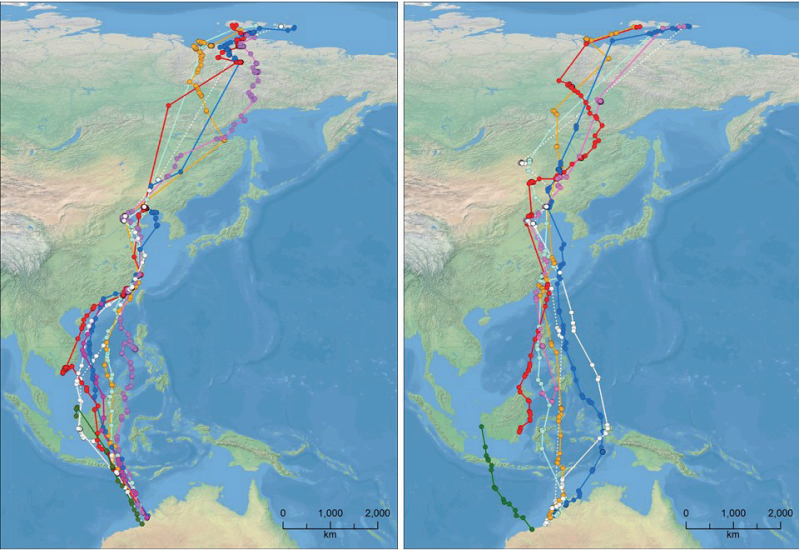
Figure 1. Individual Tracks. Tracks of the northward migration (left panel) and the southward migration (right panel) of eight individual red knots tracked from NW Australia with 2 g PTTs attached as backpacks in early 2018. The 'white' individual was tracked in both 2018 and 2019, as indicated by full and dashed lines, respectively. Dots indicate the high-quality locations used. See the summary in Table 1 for details on timing, number of stopping sites used, length of nonstop flights and detours, etc. Different colours indicate different individuals, with the track of #949 in pink, #950 in white, #953 in purple, #937 in red, #936 in blue, #905 in orange, #203 in light blue and #951 in green.

Table 1. Summary Table. Itineraries, flight lengths and detours (compared with the shortest great circle (GC) route distance) of eight red knots tracked from North West Australia (NWA) to the Yellow Sea (YS) and the New Siberian Islands (NSI) and vice versa with Microwave Telemetry solar PTTs during February 2018 – December 2019. The tracks are ordered by sex and departure date. RB=Roebuck Bay, 80MB = Eighty Mile Beach 50-55KM, ? = technical malfunction, n/a = permanent tag and/or bird failure, - = missing part of itinerary. Distances are shown in km.

PTT identifier	#949[1]	#950[2]	#950-2nd yr	#953[3]	#937[4]	#936	#905[5]	#203	#951[6]
Colouring combination	Y6LYRB	Y6RBBY		Y6LRLR	Y6RBLL	Y6RYYR	Y6LLBR	Y6LRLB	Y6RBRL
Molecular sex	M	M		M	M	M	F	F	F
Release location	RB	80MB		RB	80MB	80MB	RB	RB	80MB
Tracking year	2018	2018	2019	2018	2018	2018	2018	2018	2018
Date released Northward migration	01 March	16 Feb.		01 March	16 Feb.	16 Feb.	01 March	01 March	16 Feb.
Departure from NWA	24 April	5 May	4 May	7 May	12 May	24 May	4 May	4 May	8 May
Longest non-stop flight	4364	3359	4300	4917	3862	5467	5600	4960	3028
GC distance from NWA to first site at YS	6297	6399	6289	n/a	6527	6444	6404	6399	

Table 1. Continued.

	Individual								
PTT identifier	#949[1]	#950[2]	#950-2nd yr	#953[3]	#937[4]	#936	#905[5]	#203	#951[6]
Length of detour compared to GC	1237	906	1015	n/a	1508	938	499	493	-
Number of detected stops between NWA and first site at YS	4	2	2	>=1	2	1	1	2	-
Arrival in YS	23 May	28 May	25 May	n/a	12 June	8 June	16 May	18 May	-
Number of days in YS	9	13	13	n/a	12	15	16	15	-
Departure from YS	2 June	11 June	7 June	n/a	23 June	23 June	2 June	3 June	-
Longest non-stop flight from YS to NSI	3802	?	3788	n/a	3301	3843	4493	3515	-
GC distance from YS to first site at NSI	4007	?	4376		3916	3812	4133	3932	
Length of detour compared to GC	814	?	426	n/a	354	447	833	109	-

Table 1. Continued.

PTT identifier	Individual								
	#949[1]	#950[2]	#950-2nd yr	#953[3]	#937[4]	#936	#905[5]	#203	#951[6]
Number of detected stops between YS and NSI	1	?	1	n/a	1	1	1	1	-
Arrival at NSI	8 June	?	12 June	n.a	28 June	30 June	8 June	8 June	-
Number of days at NSI	54	?	36	n/a	31	33	49	53	-
Southward migration									
Departure from NSI	3 August	?	18 July	n/a	30 July	3 August	27 July	30 July	-
Longest non-stop flight from NSI to YS	1963	?	3200	n/a	1687	3368	3731	3088	-
GC distance from NSI to YS	4372		4019		4130	4040	3887	3974	
Length of detour compared to GC	348	?	280	n/a	925	237	252	177	-

Table 1. Continued.

PTT identifier	Individual								
	#949[1]	#950[2]	#950-2nd yr	#953[3]	#937[4]	#936	#905[5]	#203	#951[6]
Number of detected stops between NSI and YS	3	?	2	n/a	3	2	1	1	-
Arrival in YS	16 August	</= 10 Aug. [7]	30 July	n/a	18 August	16 August	31 July	8 Aug.	-
Number of days in YS	20	>/= 24	30	n/a	28	34	27	24	-
Departure from YS	6 Sept.	3 Sept.	28 Aug.	n/a	17 Sept.	21 Sept.	27 Aug.	31 Aug.	-
Longest non-stop flight from YS to NWA	2788	7096	6398	n/a	4796	5861	4914	3339	2545
GC distance from YS to NWA	n/a	6314	6344		n/a	6244	6549	6536	
Length of detour compared to GC	n/a	782	155	n/a	n/a	686	434	1050	-

Table 1. Continued.

PTT identifier	Individual								
	#949[1]	#950[2]	#950- 2nd yr	#953[3]	#937[4]	#936	#905[5]	#203	#951[6]
Number of detected stops between YS and NWA	2	0	0	n/a	1	1	1	2	-
Arrival in NWA	-	9 Sept.	3 Sept.	n/a	-	26 Oct.	12 Sept.	3 Oct.	6 Oct.

[1] Transmissions stopped on 6 November 2018 during southward migration during a stopover at Siasi Island, Sula, Philippines.

[2] In 2018 this transmitter did not transmit any locations between the birds northward departure from the Yellow Sea to its return there during southward migration. In 2019 the transmitter returned complete northward and soutward tracks.

[3] Transmission stopped during northward migration on 13 May 2018 just after what seems like a stop in the Pearl River delta between Macau and Hong Kong.

[4] Transmission stopped on 16 November 2018 during southward migration during a stopover in a bay just southwest of Balikpapan, East Kalimantan, Indonesia.

[5] Transmitter returned a full high quality track in 2018 (summarized here), and returned evidence for a return migration to the New Siberian Islands again in 2019, but with poor coverage (results not shown).

[6] Stopped during northward migration on 11 May 2018 to summer on the western tip of Kalimantan until its departure back to NW Australia on 30 October (summarized here). In 2019 this knot did a complete return migration to the New Siberian Island breeding grounds in 2019, but with minimal coverage (results not shown).

[7] Transmission recommenced on the 10th of August in the Yellow Sea during southward migration indicating that the bird arrived there at the latest

Results

Of the 18 red knots tagged in early 2018, one transmitter never sent locations, nine gave locations from the area of release but stopped transmitting before departure (of which four were later resighted in northwest Australia or China). This left us with eight satellite-tagged birds to prove that red knots from wintering grounds in NW Australia indeed migrate to the New Siberian Islands during the northern summer (Table 1, Figure 1). For most of these birds we received detailed information on their northward and southward migrations, in three cases for two successive summer seasons, yet two of these transmitters operated too intermittently for complete reconstructions of their itineraries.

The earliest departure from NW Australia occurred on 24 April, the latest on 24 May, both by males (Table 1). The earliest return to NW Australia occurred on 3 September, the latest after late October, again by males for both measures. Arrival on the New Siberian Island tundra breeding grounds occurred from 8-12 June (in two males and two females), with two other males arriving as late as 28 and 30 June. Three of the four early arriving birds (one male and two females; individuals which also left NW Australia relatively early, i.e. before 4 May) stayed long enough on the New Siberian Islands for a successful breeding season (54, 49 and 53 days, respectively). Red knots left on southward migration on 18 July (the 'repeat' male in 2019) and between 27 July and 3 August 2018 (three males and two females).

During northward migration, all final coastal staging took place in the Bohai Sea section of the Yellow Sea, i.e. north of the Shandong Peninsula in Shandong, Hebei, Tianjin and Lioaning, but never on the Korean Peninsula (Figure 2). Red knots made one to four multi-day stops *en route* and in the Yellow Sea they staged for 9-16 days (average = 13 days, Table 1). Red knots arrived in the Yellow Sea on 16 and 18

May (two females) and 23-28 May (two early males), with the two late males only arriving on 8 and 12 June. Departures occurred from 2-12 June in the two early males and two early females, but only on 23 June in the two late males. Across the six birds with complete enough itineraries, the more stops they made *en route* to the Yellow Sea, the shorter they staged there (Figure 2). The two red knots which, according to the satellite tracks, visited our red knot study area in Luannan County, were actually observed on the ground.
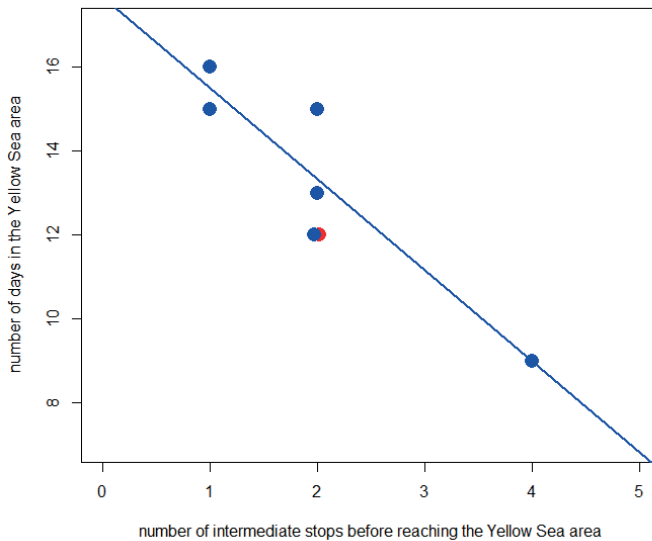
Figure 2. Stay duration and Stops. The number of refuelling days spent by individual red knots in the Yellow Sea during northward migration as a function of the number of stops made previously, *en route* between NW Australia and the Yellow Sea. The negative correlation (r= -0.92, p=0.0095) is based on the six points from 2018; see the repeat point from 2019 in red (see Table 1 for details).

During the northward migration from the Yellow Sea to the New Siberian Islands all birds made a single stop, either midway (in three cases) or on tundra on the mainland just before the sea crossing to the New Siberian Islands (two cases: the two females arriving at the breeding grounds on 8 June). Stopping also occurred during southward migration back to the Yellow Sea (up to three times, but

mostly only once, Table 1), and then always in reservoirs behind river dams or river-side lakes. Arriving back in the Yellow Sea between 30 July and 18 August, red knots then staged here for a period twice as long than during northward migration (20-34 days, average = 26 days). Departure from the Yellow Sea occurred sometime between 27 August and 21 September, with the birds returning to NW Australia either in a single non-stop flight (demonstrated three times in two males, see Table 1), or by making 1-2 stops either in Taiwan (in one case) or in the Philippines and Indonesia (three cases, two of which ended by a transmission stop, probably indicating death of the bird; Table 1).

After leaving NW Australia on northward migration, the red knots tended to keep rather westward, in most cases following either the east or west coast or crossing Borneo rather than taking the great circle route across Sulawesi (Figure 2). The birds arrived on the Chinese coast between Guandong and Fujian, rather than in the Shanghai area (Figure 2). When flying onwards to the Yellow Sea staging sites, birds kept a coastal route rather than the shortest route across land, i.e. they 'hugged' the Chinese coast. In this way they added detours between 1000 and 1500 km onto an approximately 6500 km journey (i.e. 15-25%, see numbers in Table 1). The migration paths from the Yellow Sea to the New Siberian Islands and back, and also from the Yellow Sea southward to NW Australia, did not usually precisely follow great circle paths, but were more direct than the Australia-Yellow Sea leg of their roundtrip, with the deviations mostly counting up to less than 10% of the shortest flight distance (Figure 2, Table 1). Although the four legs of the roundtrips (Australia-Yellow Sea *vice versa*, Yellow Sea-New Siberian Islands *vice versa*) only on three occasions and in two individuals was carried out as a non-stop flight, six satellite-tagged red knots showed non-stop flights over ca. 5000 km (with a maximum of 7100 km; Table 1)

Of the 21 potential red knot stopover sites, where we sampled

macrozoobenthos, 10 had high densities of the bivalve *Potamocorbula laevis* (on average, higher than 200 bivalves/m²). Probability of occurrence of red knots on these sites was higher than on the other 11 sites, with no or low densities of *Potamocorbula laevis* (X^2 = 5.67, p = 0.02, Table 2).

Discussion

In this study we have been able to confirm three inferences based on indirect observations on the occurrence and distributions of *piersmai* red knots from wintering grounds in NW Australia: (1) they do breed on the New Siberian Islands; (2) they arrive there in early June; and (3) they do stage in the Yellow Sea, but during northward migration make additional stops before arriving there. The average staging time of 13 days by satellite tagged red knots is longer than the 5-9 days estimated Lok et al. (2018) on the basis of visual observations in Luannan County, but much shorter than the three-week staging period in the Yellow Sea during southward migration.

Carrying a satellite transmitter as a backpack may come with fitness effects (Chan et al. 2016, Bodey et al. 2018). Similar patterns of flights and stops in 2012 and 2013 by five northward migrating red knots carrying 5 g solar PTTs and two carrying geolocators (Y.-C. Chan, C.J. Hassell, T. Piersma unpubl. observ.), suggest that such a handicap does not explain the pattern, and neither do the migration itineraries during southward migration, with the tagged red knots managing non-stop flights of 5000 km or more, with a maximum of 7100 km. Although the longer staging times in the Yellow Sea of the tagged birds, 9-16 days rather than the population averages of 5-9 days in Luannan county, northwest Bohai Bay (Lok et al. 2018), may be explained by the present small sample size or some geographic effect, we cannot exclude the possibility that it reflects a small degree 'handicapping'.

Table 2. Intertidel sites. Listing of intertidal sites along the coast of China used by satellite-tagged red knots on a given number of occasions in 2018 and 2019, with information on the presence of a favourite high quality food of red knots, the bivalve *Potamocorbula laevis.* Three sites with single occurrences of red knots for which we had no information on intertidal food resources were omitted.

Site (site name and province)	Latitude (°N)	Longitude (°E)	*Potamocorbula laevis* (ind./m^2)	N of tagged red knot occasions
Huanghua, Hebei Province	38.346	117.746	high density	3
Hailingdao, Guangdong Province	21.711	111.936	4369	1
Xingzhuanghe, Jiangsu Province	34.891	119.200	3816	0
Nanpu, Hebei Province	39.077	118.196	3467	1
Mutaohe, Jiangsu Province	35.013	119.212	2199	0
Shenhu, Fujian Province	24.624	118.658	1447	2
Ruian, Zhejiang Province	27.733	120.755	818	4
Nanhaipu, Shandong Province	37.459	118.942	427	0
Xinghuawan, Fujian Province	25.490	119.441	255	1
Diaokou, Shandong Province	38.089	118.578	217	0
Xiuzhenhe, Jiangsu Province	35.062	119.274	44	0
Dongliaodao, Guangdong Province	20.825	110.384	17	0
Cixi, Zhejiang Province	30.396	121.194	7	0
Changyi-W, Shandong Province	37.146	119.406	5	0

Table 2. Continued.

Site (site name and province)	Latitude (°N)	Longitude (°E)	*Potamocorbula laevis* (ind./m²)	N of tagged red knot occasions
Panjin, Liaoning Province	40.606	122.046	2.5	3
Xinchuangang, Jiangsu Province	32.627	120.989	1	0
Yalujiang, Liaoning Province	39.804	123.926	0	0
Changyi-E, Shandong Province	37.129	119.572	0	0
Tongzhou, Jiangsu Province	32.177	121.430	0	0
Qidong, Jiangsu Province	32.003	121.775	0	0
Raoping, Guangdong Province	23.593	117.142	0	1

Surprisingly, during the first leg of the northward migration, rather than making a straight northward flight to the Yellow Sea (e.g. by #949 indicated by the track in pink, Figure 1), most red knots took quite a detour, 'hugging' the Southeast Asian mainland and especially the Chinese coast. As they did not make stops 'at the earliest convenience', i.e. stopping at the first possible site in southern China, the early landfall of the birds does not seem to indicate them running out of energy. In view of the general capacity of red knots to migrate across large swaths of inhospitable terrain (as they do during the flights across the Asian landmass to and from the New Siberian Islands, Figure 1; and see e.g. Niles et al. 2010, Newstead et al. 2013, Kok et al. 2020b for similar feats in other subspecies including the 8100 km nonstop flight reported at https://whsrn.org/uncovering-the-mysteries-of-red-knot-movements-on-the-gulf-coast/#), the absence of inland eastern China crossings is puzzling. The 'tracing' of the Chinese coastline is probably the result of good navigational or ecological reasons, which

may include the possibility to collect information on the quality of staging areas along the way.

Stopovers mostly occurred at sites where our sampling of parts of the local intertidal areas showed high densities of *Potamocorbula laevis* (Table 2), a strongly preferred and high-quality prey type for both red knots (Yang et al. 2013, 2016) and the similarly molluscivorous great knot *Calidris tenuirostris* (Choi et al. 2017, Zhang et al. 2019a,b). Although this has not been documented in the field, during the three-week southward staging bouts in the Yellow Sea, the red knots are likely to fuel up again on a diet of *Potamocorbula*, a prey species that seems key to the success and survival of several shorebird species (e.g. Choi et al. 2017, Zhang et al. 2018, Chan et al. 2019a).

Although the length of non-stop flights are of the same magnitude as in other subspecies of red knots, we must nevertheless concede that, during their northward migration from northwest Australia to the Yellow Sea, *piersmai* red knots behave as 'skippers' rather than 'long-jumpers' (Piersma 1987). This pattern of making multiple stops of about one week (Lok et al. 2018), appears spatially correlated with the presence of suitable staging habitats along the Southeast Asian coastline from Vietnam to the China-North Korea border in the Yellow Sea. The coastal sites along the coast of China where red knots stopped or staged were characterized by the presence of high densities of what can be regarded as a super-food for red knots, the bivalve *Potamocorbula laevis* (Table 2). This may suggest that along the East Asian-Australasian Flyway red knots actually have more staging opportunities than along other flyways (Piersma et al. 2005).

Is the conclusion of the availability of multiple potential staging habitats along part of the flyway at odds with the finding that the red knots of the East Asian-Australasian Flyway, both the subspecies *piersmai* and *rogersi*, have actually been in decline for at least a decade (Conklin et al. 2014, Piersma et al. 2016, Studds et al. 2017)? Have red knots always used multiple staging sites during the first leg of the northward

migration, or is this pattern a consequence of the recent reductions in the extent of suitable habitat in the Yellow Sea area (Murray et al. 2014, Piersma et al. 2016), reductions which in the remaining habitats along the Luannan coast in the western part of Bohai Bay seems to have led to a concentration of staging red knots (Yang et al. 2011)? The tendency to start land claims at the valuable and much used upper parts of intertidal soft sediment systems, certainly will have continued to reduce the extent of foraging opportunities of red knots along the coast of China over the last 15 years (Mu and Wilcove 2020).

Acknowledgements

CHAPTER 4

EVOLUTIONARY DESIGN OF A FLEXIBLE, SEASONALLY MIGRATORY, AVIAN PHENOTYPE: WHY TRADE GIZZARD MASS AGAINST PECTORAL MUSCLE MASS?

Kimberley J. Mathot, Eva M.A. Kok, Joseph B. Burant, Anne Dekinga, Petra Manche, Darren Saintonge and Theunis Piersma

Migratory birds undergo impressive body remodelling over the course of an annual cycle. Prior to long-distance flights, red knots (*Calidris canutus islandica*) reduce gizzard mass while increasing body mass and pectoral muscle mass. Although body mass and pectoral muscle mass are functionally linked via their joint effects on flight performance, gizzard and pectoral muscle mass are thought to be independently regulated. Current hypotheses for observed negative within-individual covariation between gizzard and pectoral muscle mass in free-living knots are based on a common factor (e.g., migration) simultaneously affecting both traits, and/or protein limitation forcing allocation decisions. We used diet manipulations to generate within-individual variation in gizzard mass and test for independence between gizzard and pectoral muscle mass within-individuals outside the period of migration and under conditions of high protein availability. Contrary to our prediction, we observed a negative within-individual covariation between gizzard and pectoral muscle mass. We discuss this result as a potential outcome of an evolved mechanism underlying body remodelling associated with migration. Although our proposed mechanism requires empirical testing, this study echoes earlier calls for greater integration of studies of function and mechanism, and in particular, the need for more explicit consideration of the evolution of mechanisms underlying phenotypic design.

Introduction

Understanding the causes and consequences of phenotypic variation is a central aim in ecology and evolution (West-Eberhard 2003, Piersma and van Gils 2011). Organisms are made up of multiple traits which show varying degrees of inter-dependence. To account for this complexity, it is becoming increasingly common for researchers to adopt a multi-trait approach to understanding phenotypic variation (Badyaev et al. 2001, Badyaev and Young 2004, Young and Badyaev 2006, Araya-Ajoy and Dingemanse 2014, Dillard and Westneat 2016). Positive covariances between traits are suggestive of functional integration (i.e., phenotypic integration) (Pigliucci 2003, Armbruster et al. 2014), while negative covariances are suggestive of trade-offs (Stearns 1989). For example, flight performance in birds is determined by overall body mass and the size of the pectoral muscle (Pennycuick 1969, 1975). All else being equal, birds that are heavier require larger pectoral muscles to achieve the same flight performance as lighter birds (Pennycuick 1975, 1989, Piersma and Dietz 2007). This functional association between body mass and pectoral muscle mass manifests as a positive covariance between the two traits (e.g., Lindström et al. 2000, Dietz et al. 2007, van den Hout et al. 2010). Traits can also be linked via trade-offs when organisms have limited resources that must be allocated to competing processes (Stearns 1989, Dietz et al. 2007, Dietz and Piersma 2007, Vézina et al. 2010). For example, under conditions with limited access to dietary protein, higher protein investment towards one muscle group must come at the cost of investment in another muscle group (Bauchinger and Biebach 1998, 2001, Hua et al. 2013).
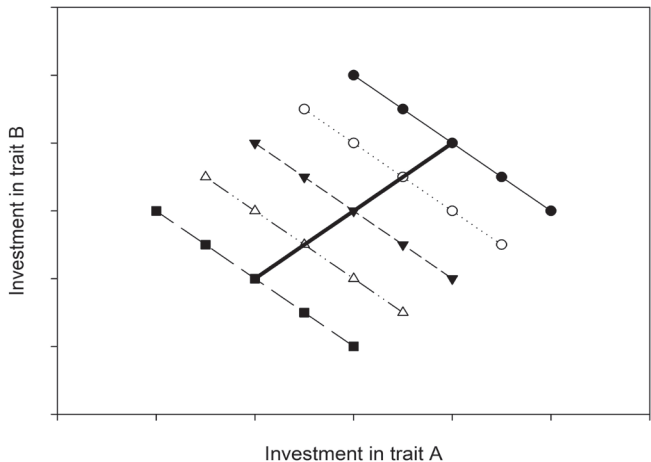
Figure 1. Illustration of trait covariances. Illustration of trait covariances that differ at different levels of organization. Individuals are represented by different symbols and each individuals is represented by 5 points and a thin line. At the within-individual level, a higher investment in trait A is associated with a lower investment in trait B. However, across individuals, the relationship is reversed. Individuals that have a higher average expression of trait A also have a higher average expression of trait B. The thick black line shows the relationship across the average values for each individual.

For labile phenotypic traits, patterns of covariation between traits can vary across different levels of organisation (e.g., within- versus among-individuals; Westneat et al. 2015). For example, when resources (e.g., time or energy) are limited, the more an individual invests in trait A, the less resource is available to invest in trait B. This generates negative covariation between traits A and B at the within-individual level. However, individuals may differ in how much resource they have available to them, for example, because some individuals are on higher quality territories than others. Individuals with access to more resource can invest more on average in both trait A and trait B (van Noordwijk and de Jong 1986). Individual differences in access to resource shape

positive correlations between traits A and B at the among-individual level (Figure 1). When the relative importance of different mechanisms in shaping patterns of covariation differs at the within- versus among-individual level, un-partitioned phenotypic correlations can obscure level-specific relationships (van Noordwijk and de Jong 1986, Dingemanse and Dochtermann 2013, Metcalf 2016). Thus, studying the covariance structure of multiple traits and partitioning phenotypic covariations to the within- and the among-individual levels can provide insights regarding level-specific mechanisms shaping patterns of trait co-variation (Dingemanse and Dochtermann 2013, Araya-Ajoy et al. 2016, Dingemanse 2017, Iserbyt et al. 2017). However, studying trait integration at the within-individual level requires study systems for which it is possible to repeatedly measure the traits of interest in the same individual (Dingemanse and Dochtermann 2013). Although this is relatively straightforward for behavioural traits and certain physiological traits (e.g., metabolic rates, hormone levels, etc.), studies on integration of internal structures (e.g., organ size) are rare because of the methodological limitations of repeatedly assaying such traits in the same individuals (Lindström and Piersma 1993, Piersma and Lindström 1997, Dietz et al. 1999a). Red knots (*Calidris canutus*), a medium-sized migratory shorebird, provide a rare opportunity to study level-specific trait covariation of internal traits, because non-destructive methods have been developed that allow for accurate estimation of internal organs (Dietz et al. 1999a). Red knots have also served as good experimental animals to test for seasonally changing phenotypic traits that are related to migration but are maintained in captivity (Dietz et al. 1999b, Karagicheva et al. 2016), together with the bodily consequences of differences in ecological contexts such as ambient temperature (Vézina et al. 2006, 2017), predation risk (van den Hout et al. 2010) and diet (Gutiérrez et al. 2015, this study).

In this study, we focus on three ecologically important traits for knots: body mass (size, storage capacity, resource holding potential; Piersma

and Davidson 1991), pectoral muscle mass (flight performance; Pennycuick 1975, 1989, Dietz et al. 2007, van den Hout et al. 2010), and gizzard mass (digestive performance; van Gils et al. 2003, 2005b). At the among-individual level, we predicted positive covariance between all three traits if they are simple expressions of body size. At the within-individual level, we predicted different trait covariance structures. Body mass and pectoral muscle mass are functionally linked through their concurrent effects on flight (van den Hout et al. 2006), and previous studies in free-living knots have reported positive within-individual covariance between these two traits (e.g., Lindström et al. 2000). In contrast, gizzard mass and pectoral muscle mass are likely independently regulated traits. Nevertheless, a negative within-individual covariance was reported between body mass and gizzard mass (Piersma et al. 1999a, Dietz et al. 1999b, Vézina et al. 2012), and between pectoral muscle mass and gizzard mass (Piersma et al. 1999b, Dietz et al. 1999b, Vézina et al. 2012) as a result of body remodeling associated with migration. These patterns are similar to patterns that have been observed in other birds (Lindström and Piersma 1993, Piersma and Lindström 1997, Bauchinger and Biebach 1998, 2005, Biebach and Bauchinger 2003, Landys-Ciannelli et al. 2003, Piersma and van Gils 2011). Current hypotheses for the observed negative covariation between gizzard mass and pectoral muscle mass in free-living knots are based on a common factor (i.e., migration) simultaneously affecting both traits (Piersma and Lindström 1997, Biebach and Bauchinger 2003, Bauchinger et al. 2005, Vézina et al. 2012, Hua et al. 2013), protein limitation forcing allocation decisions between gizzard mass and pectoral muscle mass (Bauchinger and Biebach 1998, 2001, Vézina et al. 2012), or a combination of the two. Thus, outside the migratory season and under conditions of high protein availability, gizzard mass and pectoral muscle mass are predicted to vary independent of one another at the within-individual level.

We used diet manipulations to generate within-individual variation in gizzard mass and test for the independence of gizzard mass and pectoral muscle mass at the within-individual level. Knots are mollusc eating shorebirds that swallow their prey whole (Piersma 2012). Prey are crushed in the muscular stomach (i.e., gizzard). Gizzard mass is flexible, individuals adjust their gizzard mass over the course of days in response to changes in the digestive quality (i.e., the ratio of digestible to indigestible part) of their diets such that when individuals consume a diet of high digestive quality (i.e., with a high ratio of digestible to indigestible parts) they develop smaller gizzards, and when they consume a diet of low digestive quality they develop large gizzards (Piersma et al. 1993b, Dekinga et al. 2001, Battley and Piersma 2005a). Manipulations of gizzard mass were performed over two successive non-breeding seasons, under conditions of *ad libitum* access to protein-rich food. We predicted that experimental manipulation of gizzard mass would not induce any systematic change in pectoral muscle mass.

Methods

Study subjects

Red knots of the *islandica* subspecies (see e.g., Piersma 2007) were captured using mist nests on the mudflats of Richel (53°16'N, 05°23'E), Griend (53°14'N, 05°15'E) and Schiermonikoog (53°28'N, 06°10'E) in the Wadden Sea, The Netherlands, between August and October 2015 and transported to the experimental shorebird facilities at the NIOZ Royal Netherlands Institute for Sea Research. Outside of the experiments (April through October), birds were housed in 8 aviaries in flocks of between 14 and 17 individuals, and were maintained on an *ad libitum* diet of protein-rich trout pellets (Trouvit, Produits Trouw, Vervins, France). Birds were handled each week to assess overall health (Milot et al. 2014) and to allow the aviaries to be cleaned. Further details on husbandry conditions are provided in ESM Text S1.

Experimental procedure

Experiments were conducted between October and March in each year, which is outside the migration period of *islandica* red knots (Dietz et al. 2007). During this time, birds experienced eight diet manipulations (four in each year, see below) with concurrent measurements of body mass, gizzard mass and pectoral muscle mass. Diet manipulations were used to generate within-individual variation in gizzard mass (Dekinga et al. 2001). To induce small gizzards, knots were maintained on an *ad libitum* diet of Trouvit, the same high digestive quality food as provided outside of the experiments. Trouvit pellets are 47% protein by dry weight. To induce large gizzards, knots were fed an *ad libitum* staple diet of thawed mudsnails, *Peringia ulvae* (formerly *Hydrobia ulvae*). Knots offered a staple diet of *Peringia* consume them whole, thereby ingesting a large fraction of indigestible material. Thus, *Peringia* is of low digestive quality and induces gizzard hypertrophy (e.g., van Gils et al. 2005b, Vézina et al. 2011, Mathot et al. 2017). Although we did not obtain measures for the protein content of the *Peringia* used in our experiments, molluscs are generally recognized as being a protein-rich food (range 50% to >80% protein by dry weight) (Sarvaiya 1989, Nie et al. 2016, Ab Lah et al. 2017). Further, in other studies, knots have been kept on diets of *Peringia* for extended periods (3 to 12 months) (Mathot et al. 2009, van den Hout et al. 2010, Vézina et al. 2011), with no evidence of loss of body condition, as would be expected if this food was low in protein. This was also true in the present study; knots were maintained on *Peringia* diets repeatedly for up to 6 consecutive weeks with no evidence of loss of body condition (personal observation).

In each year of the experiments, all individuals were subjected to two replicates of the *Peringia* diet treatment (P) and two replicates of the Trouvit diet treatment (T) in alternating sequence. Half of the aviaries (and therefore half of the birds) were randomly assigned to the PTPT treatment order, and the remaining half of aviaries to TPTP treatment order. By using a balanced crossover design, we ensured that

experimentally induced variation in gizzard mass was not confounded with any circannual endogenous rhythms in any of the three focal traits (Battley and Piersma 2005b, Karagicheva et al. 2016).

Previous work has shown that gizzard mass adjusts to the digestive quality of the diet within 6 to 14 days (Dekinga et al. 2001). To ensure that gizzard mass was stable over the duration of physiological measurements in a given treatment block, we allowed birds to remain on staple diets for at least three weeks before commencing physiological measurements. Gizzard mass and pectoral muscle mass were measured by AD using an ultrasound scanner (model Aquilla, Pie Medical Benelux, Maastricht, The Netherlands). Detailed descriptions of the procedure are provided elsewhere (Dietz et al. 1999a, Dekinga et al. 2001, ESM Text S2). Following physiological measurements, birds were subjected to behavioural observations as part of another study (Kok et al. 2019a). These observations required an additional three weeks on the staple diet, resulting in up to 6 consecutive weeks on any given diet treatment.

Data selection and statistical analyses

Some knots did not experience the experimentally determined diet manipulation sequence because some individuals did not adjust to switches to a *Peringia* diet (i.e., the low digestive quality diet) (n=3). These individuals were excluded because their physiological trait values may have been in flux (see Dietz et al. 2013). Similarly, birds with *Staphylococcus* infections had missing observations as we did not measure trait values in birds that were being treated for active infections (n = 5 cases). Thus, the final sample size for the analyses presented here are: N =584 observations, N = 88 individuals.

Our experimental subjects included birds caught as juveniles (N = 44) and birds captured as adults (N = 44) and spanned two successive non-breeding seasons. We considered the possibility that covariance structures would be year- or cohort-specific, and therefore initially

ran four separate multivariate models. However, there was no support for differences between the covariance matrices (see ESM Table S1). Therefore, for simplicity, analyses presented in the main text are from a single multivariate model, including data from both years and both age cohorts.

We estimated the among- and within-individual correlations by fitting gizzard mass, pectoral muscle mass, and body mass as three response variables using the *MCMCglmm* function (Hadfield 2010) in the R statistical environment (R Core Team 2017) and modelling random intercepts for bird identity. We did not include random intercept for aviary identity because univariate analyses revealed this to be of minor importance for all three traits (analyses not shown). See ESM Text S3 for further details on MCMCglmm implementation procedures.

Because total body mass (BM) includes gizzard mass and pectoral muscle mass, positive correlations may have resulted from part-whole correlations (see Christians 1999) for discussion). We addressed this possibility by repeating analyses using body mass minus pectoral muscle mass to estimate correlations between pectoral mass-independent body mass and pectoral muscle mass, or body mass minus gizzard mass to estimate correlations between gizzard mass-independent body mass and gizzard mass. We compared the results of these analyses with results from analyses using overall body mass and present both.

We predicted positive among-individual covariance between body mass, gizzard mass and pectoral muscle mass if each of these traits are expressions of overall body size. To further assess this possibility, we tested whether an individual's average gizzard mass, average body mass and average pectoral muscle mass correlated with their structural body size. For 68 individuals, we had complete data on three measures of structural body size (bill length, wing length and tarsus length), as well as sufficient measures of body mass, gizzard mass

and pectoral muscle mass (minimum four per individual, two on each diet treatment) to calculate an individual's average value for each of these traits. Following earlier studies (Bijleveld et al. 2014, Mathot et al. 2017), we use the first principal component (PC1) of a principal component analysis of wing length, tarsus length and bill length as our measure of structural body size using the *prcomp* function. Next, we estimated pairwise correlations (estimate and 95% CI) between average body mass, average gizzard mass, and average pectoral muscle mass using the *corr.test* function. Because our study design prevented any confounding relationship between time of year (e.g., relative to migration, photoperiod, temperature, etc.) and gizzard mass, within-individual relationships between gizzard mass and pectoral muscle mass or body mass could not be due to time of year effects, and therefore, we did not correct for time of year in these analyses.

Next, we evaluated the amount of within-individual variance for each of our three focal traits (gizzard mass, pectoral muscle mass, and body mass) that could be explained by our experimental diet treatments. To do this, we constructed univariate mixed effects models with either gizzard mass, pectoral muscle mass or body mass as a response variable, and diet treatment as a fixed effect. Bird id was included as a random effect. We then used the *r.squaredGLMM* function from the 'MuMIn' package in R which calculates the percent of variation in the response variable explained by fixed effects.

Finally, we estimated the slopes of the within- and among-individual correlations using two datasets: an among-individual data set, and a within-individual data set. The among-individual data set was comprised of average trait values per individual estimated across all repeated measures. The within-individual data set was comprised of within-individual centered data in which each individual's mean trait value was subtracted from each observation for that individual (van de Pol and Wright 2009). As all variables have associated measurement error, we used reduced major axis (RMA) regression to estimate slopes

between pairs of traits using the *lmodel2* function from the 'lmodel2' package.

For all analyses, we evaluated support for effects based on estimated effects sizes and their 95% credible intervals (CIs; Cumming and Finch 2005, Nakagawa and Cuthill 2007). This approach has been advocated to avoid drawing dichotomous conclusions to accept or reject the null hypothesis based on data which can show a continuous range of support (or lack of support) for a given interpretation (Cohen 1990, Nakagawa and Cuthill 2007, Wasserstein and Lazar 2016). However, for readers less familiar with the use of CIs, a 95% CI that does not overlap zero is roughly equivalent to a significant p-value in the frequentist's sense (Cumming and Finch 2005), we describe such results as showing 'strong support' for predictions. For estimates that are biased away from zero but whose 95% CIs overlap zero by up to 15%, we use the term 'moderate support'. For estimates centered on zero with 95% CIs greatly overlapping zero we use the term 'no support for an effect' or the term 'strong support for lack of effect'. We used visual assessment of the residuals to evaluate model fit. The strength of correlations was described as either weak ($|r| < 0.3$), moderate ($0.3 < |r| < 0.5$), or strong ($|r| > 0.70$).

Results

There was substantial variation in all three traits, both in the average values per individual (i.e., the amount of among-individual variation), and in the range of values expressed per individual (i.e., the amount of within-individual variation). The among-individual variation in body mass ranged from 116 to 186 g, pectoral muscle mass ranged 24.0 to 33.9 g, and gizzard mass ranged from 4.64 to 8.26 g). As predicted, there was strong support for positive correlations between all these traits at the among-individual level (95% CIs did not overlap with 0), and these correlations were all strong (all $|r| > 0.70$) (Table 1, left panel of Figure 2). Analyses correcting for the effect of pectoral muscle mass and

gizzard mass on total body mass yielded quantitatively similar results (Table 1, values in italicized bold font), indicating that the among-individual correlations between gizzard mass overall body mass, and between pectoral muscle mass and overall body mass, were not solely due to part-whole correlations. Additionally, there was moderate (e.g., for gizzard mass and pectoral muscle mass) to strong support (e.g., for body mass) that all three traits were positively correlated with structural body size (Table 2).

The average within-individual variation in body mass was 29 g (range: 4 to 115 g), pectoral muscle mass was 6.6 g (range 0.8 to 13.9 g), and gizzard mass was 5.2 (range: 1.4 to 8.2 g) in the course of the study. As expected, a large proportion of the within-individual variation in gizzard mass could be explained by diet treatment ($R^2 = 45.54\%$). However, the proportion of within-individual variation in pectoral muscle mass and body mass that could be explained by the diet treatment was markedly lower (2.37% and 0.33% respectively), indicating that additional, non-experimental factors, contributed substantially to the within-individual variation in those traits (e.g., ambient temperature; Vézina et al. 2011). At the within-individual level, there was strong support for positive correlations between both gizzard mass and body mass, and between pectoral muscle mass and body (Table 1).

The strength of the correlation between body mass and pectoral muscle mass was strong ($|r| > 0.5$), while the strength of the correlation between gizzard mass and body mass was weak ($|r| > 0.10$). We also found moderate support for a weak ($|r| < 0.30$) negative within-individual correlation between gizzard mass and pectoral muscle mass (Table 1). When correcting for part-whole correlations, there was strong support for a positive within-individual correlation between pectoral muscle mass and body mass minus pectoral muscle mass of moderate strength. In contrast, there was no support for a correlation between gizzard mass and body mass independent of gizzard mass (Table 1).

Table 1. Covariance matrix for body mass, pectoral muscle mass, and gizzard mass in red knots. Values above the diagonal are among-individual correlations, values below are within-individual correlations. Estimates presented in bold italics are from identical models run using body mass independent of the muscle type for which the correlation is estimated (e.g., for correlation between body mass and gizzard mass, gizzard mass is subtracted from total body mass).

	Body mass (g)	Pectoral muscle mass (g)	Gizzard mass (g)
Body mass (g)	-	0.79 (0.67, 0.87) *0.56 (0.51, 0.62)*	0.85 (0.63, 0.97) *0.85 (0.63, 0.98)*
Pectoral muscle mass (g)	0.54 (0.48, 0.60) *0.36 (0.29, 0.44)*	-	0.79 (0.53, 0.96)
Gizzard mass (g)	0.13 (0.04, 0.21) *-0.03 (-0.11, 0.05)*	-0.13 (-0.22, 0.05)	-
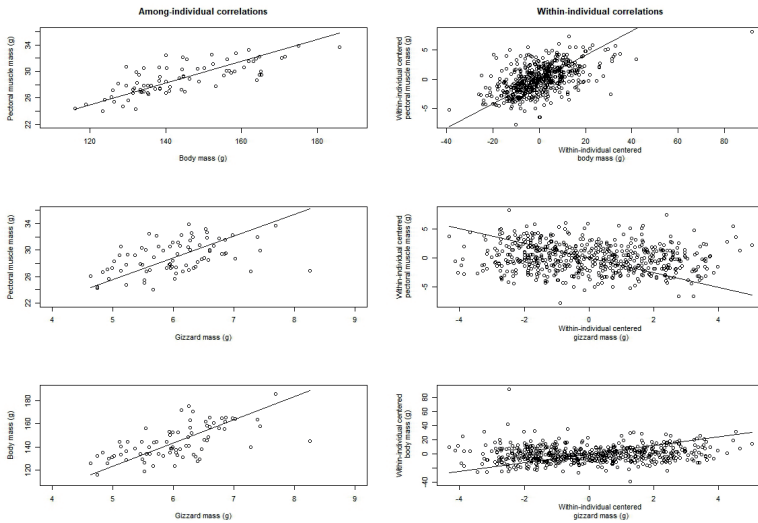
Figure 2. Among-individual (left panel) and within-individual (right panel) correlations between gizzard mass (g), pectoral muscle mass (g), and body mass (g). For the among-individual correlations, each circle represents the average value per individual (mean of between 4 and 8 measurements per individual). For the within-individual correlations, each individuals average trait value was subtracted from each measure, thus, values represent deviations from the individual's average, and each individual is represented by between 4 and 8 data points in each figure in the left panel. Solid lines show the slopes of a reduced major axis (RMA) regression.

The results of the RMA regression analyses show that the slope of the relationship between body mass and pectoral muscle mass was quantitatively similar at the among- and within-individual level (see Figure 2 and ESM Table S2). At the among-individual level, a 1 g increase in body mass was associated with a 0.16 g (95% CI: 0.14, 0.19) increase in pectoral muscle mass, while at the within-individual level, 1 g increase in body mass was associated with a 0.21 g (95% CI: 0.20-0.22) increase in pectoral muscle mass. The slope between overall body mass and gizzard mass tended to be steeper at the

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within-individual level compared with the among individual level. At the among individual level, for every 1 g increase in overall body mass, there was a 0.05 g (95% CI: 0.04, 0.06) increase in gizzard mass, compared with 0.17 g (95% CI: 0.15, 0.18) increase at the within-individual level. The slope between gizzard mass and pectoral muscle mass was of similar magnitude, but in the opposite direction, at the among- versus within-individual level. At the among-individual level, for every 1 g increase in gizzard mass, there was a 3.23 g (95% CI: 2.68, 4.01) increase in pectoral muscle mass, versus a 1.26 g decrease (95% CI: -1.37, -1.16) at the within-individual level.

Discussion

We used a multivariate approach to study trait integration at the among- and within-individual level in red knots for three ecologically important traits: body mass, gizzard mass and pectoral muscle mass. Covariation between body mass and pectoral muscle mass, and between gizzard mass and body mass, was positive at both the among- and within-individual levels. Heavier knots had heavier pectoral muscles and gizzards, and when individual knots become heavier, both their pectoral muscle and gizzard mass increased. However, the relationship between gizzard mass and pectoral muscle mass was level-specific: there was a strongly positive correlation at the among-individual level indicative of trait integration, but a weakly negative correlation at the within-individual level indicative of a trade-off. The negative within-individual covariation between gizzard mass and pectoral muscle mass suggests that these two traits are not independently regulated. We propose that their negative within-individual covariance may be the outcome of an evolved mechanism underlying body remodelling associated with migration.

Table 2. Pearson's Correlations. Results of Pearson's correlations between structural body size (PC1 of wing length, bill length and tarsus length) and average body mass, average gizzard mass, and average pectoral muscle mass. Data from N = 68 individuals.

Pearson's correlation between PC1 and:	r (95% CI)
Average body mass	0.33 (0.09, 0.52)
Average gizzard mass	0.16 (-0.09. 0.38)
Average pectoral muscle mass	0.23 (-0.01, 0.44)

If body mass, gizzard mass and pectoral muscle mass are all expressions of overall body size, there should be positive among-individual correlations between all three traits. Indeed, in addition to being positively correlated with each other, each of these traits also tended to be positively associated with measures of structural body size (e.g., tarsus length, wing length, bill length, Table 2). Structurally larger knots were heavier, had larger gizzards and larger pectoral muscles. However, we also observed positive correlations between gizzard mass and body mass, and between pectoral muscle mass and body mass at the within-individual level, suggesting that associations between these traits occur independently of overall body size (note that structural body size does not vary at the within-individual level, because mature individuals do not change in structural body size; Piersma and Davidson 1991).

The positive covariation between total body mass and pectoral muscle mass at both the among- and within-individual levels can be understood from the functional association that each of these traits has with flight performance (Pennycuick 1975). Although the strength of the covariation was slightly lower at the within-individual level (Table 1), the estimated increase in pectoral muscle mass per 1 g increase in body mass was quantitatively similar at both the among- and within-individual levels (ESM Table S2), suggesting that the covariation between total body mass and pectoral muscle mass is shaped by the same mechanism at both these levels: power output (Dietz et al. 2007).

The covariation between total body mass and gizzard mass was also positive at both the among- and within-individual levels; however, the patterns were quantitatively different. At the among-individual level, the covariation was strong, while at the within-individual level the covariation was weak. We suggest that the differences in the strength of the covariation between gizzard mass and overall body mass across these two levels reflects differences at the within- and among-individual level in the mechanisms generating integration between these traits. Consistent with this interpretation, covariation between body mass after correcting for part-whole correlations (by subtracting the mass of the gizzard) and gizzard mass remained strong and positive at the among-individual level, but disappeared at the within-individual level. We interpret the integration between body mass and gizzard mass at the among-individual level as reflecting body size variation, with larger birds being heavier and also requiring larger digestive organs to fuel their higher energy demands. In contrast, the within-individual correlations can be fully explained by part-whole correlations, suggesting a lack of integration between the traits at this level.

Several empirical studies previously have documented a negative within-individual correlation between gizzard mass and pectoral muscle mass (Piersma et al. 1999a,b, Dietz et al. 1999b, Vézina et al. 2012). This negative covariation has been suggested to result from a common factor, migration, exerting opposing effects on two independent traits (Piersma and Lindström 1997, Biebach and Bauchinger 2003, Bauchinger et al. 2005) and/or limited access to protein creating a trade-off between investment in gizzard muscle versus pectoral muscle (Bauchinger and Biebach 1998, 2001). Despite the fact that we conducted the experiments outside the migration periods and under conditions of unlimited access to high protein diets, we observed the within-individual negative covariation between gizzard mass and pectoral muscle mass. Thus, our results demonstrate

that neither migration nor protein limitation are required to generate this trade-off. One possible explanation for the apparent trade-off is that the requirements to build up gizzard mass on low digestive quality diets exceeded the capacity to assimilate endogenous protein, which could result in reallocation of pectoral muscle protein even under conditions of high dietary protein. When low digestive quality diets necessitate a rapid build up of gizzard mass, for every 1 g increase in gizzard mass, knots atrophied their pectoral muscles by 1.3 g (ESM Table S2). Previous work has shown that knots can fully adjust their gizzards to changes in the digestive quality of their diets within six days (Dekinga et al. 2001). Given that the knots in the present study had at least three weeks on any diet treatment, this would have allowed two additional weeks to rebuild pectoral muscle mass after adjusting their gizzards. We did not quantify protein assimilation in this study, and therefore, we cannot rule out the possibility that the negative within-individual covariation between gizzard mass and pectoral muscle mass reflects a constraint on protein update. However, we suggest this is unlikely to account for our findings because typical digestion efficiencies of protein by birds are in excess of 50% (Castro et al. 1989, Moughan et al. 2014). Further, there was no evidence of protein limitation during our study given that knots maintained good body condition throughout the experiments (personal observation).

We suggest that an exclusive focus on the functional significance of gizzard mass and pectoral muscle mass is insufficient to understand the observed pattern of covariation. From a functional perspective, in the context of these experiments, there is no reason why developing a larger gizzard would be predicted to favour atrophy of pectoral muscle mass, or *vice versa*. A more complete understanding of patterns of within-individual trait covariances requires explicit consideration of the evolution of the mechanisms mediating trait expression (sensu McNamara and Houston 2009). We suggest that the observed negative within-individual correlation between gizzard mass and pectoral

muscle mass may provide insights into the evolved mechanism underlying migration-related body remodelling in red knots.

In free-living knots, a negative within-individual covariation between gizzard mass and pectoral muscle mass is adaptive. During migration, knots benefit from reducing gizzard mass because it does not serve a function during fasting associated with long-distance flights and is costly to maintain, while at the same time they benefit from developing large pectoral muscles to power flights (Piersma et al. 1999b). If large and rapid changes in gizzard mass in free-living knots are reliably associated with conditions that favour simultaneous and opposing changes in pectoral muscle mass, we suggest that this may have led to the evolution of a mechanism whereby large, rapid changes in gizzard mass trigger opposite changes in pectoral muscle mass.

Do large and rapid changes in gizzard mass reliably coincide with migration? Although gizzard mass responds to variation in diet over short-time scales (i.e., approximately 1 week for 2-fold adjustment; Dekinga et al. 2001), this magnitude and rate of change has been documented in captive studies in which knots were subjected to large and absolute changes in the quality of their diets (e.g., shift from exclusively high digestive quality diet to exclusively low digestive quality diet). In free-living knots, day to day variation in the quality of encountered prey would neither be as extreme in magnitude, nor as absolute. Even under conditions of starvation, knots defend their gizzard mass at the expense of other lean body components (including pectoral muscle; Dietz and Piersma 2007). Therefore, changes in gizzard mass outside of the migration period are unlikely to be extreme, and the most predictable occurrence of large and rapid changes in gizzard mass are likely to be those observed prior to long-distance flights (e.g., Piersma et al. 1993b, Piersma and Lindström 1997, Battley and Piersma 1997), when opposing changes in pectoral muscle would indeed be favoured.

We propose that the mechanism underlying migration-related body remodelling operates unidirectionally. That is, large and rapid changes in gizzard mass induce opposing changes in pectoral muscle mass, but not *vice versa*. The reasons for this are two-fold. First, this order of organ adjustment is adaptive in the context of migratory remodelling. In free-living shorebirds, gizzard mass atrophy prior to long-distance flights precedes pectoral muscle mass hypertrophy by more than a week (Piersma et al. 1999b, Landys-Ciannelli et al. 2003, Hua et al. 2013). From an energy management perspective, it seems adaptive to delay the build-up of pectoral muscle mass until shortly before migration given the high metabolic cost of the flight muscles (Vézina et al. 2017), although we cannot exclude the possibility that this pattern reflects an unknown constraint. Second, while large, rapid changes in gizzard mass may be predictably associated with migration (see above), the same is not true for large and rapid changes in pectoral muscle mass. Pectoral muscle mass decreases rapidly under starvation (Dietz and Piersma 2007), increases rapidly in response to low temperatures (Vézina et al. 2006, 2007, 2011), and closely tracks overall body mass (Lindström et al. 2000). Thus, rapid changes in pectoral mass cannot serve as a reliable indicator of migration status and therefore cannot be used to direct migration-related changes in gizzard mass. Consistent with this notion, experiments in knots that induced larger pectoral muscles by exposing them to colder ambient conditions found no coincident changes in gizzard mass (Vézina et al. 2011).

Although numerous studies have experimentally generated rapid changes in gizzard mass in knots (Dekinga et al. 2001, Bijleveld et al. 2014, Oudman et al. 2016, Mathot et al. 2017), we are aware of only one other study in which pectoral muscle mass was measured simultaneously. In that study, knots were given a staple diet of high digestive quality following capture, resulting in a large (50%) decrease in gizzard mass (Dietz et al. 1999b). Over the same time

period, pectoral muscle mass increased 20%, with almost no change in overall body mass (Dietz et al. 1999b). Although these results are consistent with our proposed mechanism, the timing of gizzard and pectoral muscle measurements, as in the present study, was too coarse to elucidate the timing of changes in pectoral muscle mass relative to gizzard mass. If our proposed mechanism is correct, we would predict that diet-induced changes in gizzard mass precede changes in pectoral muscle mass. This is corroborated by our finding that diet explains a large proportion of the within-individual variation in gizzard mass, while within-individual variation in gizzard mass explains more of the within-individual variation in pectoral muscle mass than does diet. Nonetheless, studies measuring diet-induced changes in gizzard mass and pectoral muscle mass on a finer temporal scale would provide stronger tests of this idea. Further, we would predict that smaller and/ or slower changes in gizzard mass (e.g., changes induced by partial changes in diet) would not be sufficient to trigger changes in pectoral muscle. This is because small and/or slow changes in gizzard mass are likely to occur throughout the annual cycle of knots, when coincident changes in pectoral muscle mass are not necessarily beneficial. A mechanism whereby pectoral muscle mass would continuously adjust to small within-individual variations in gizzard mass would therefore not be beneficial.

Conclusion

Many long-distance migrants undergo dramatic body remodelling associated with the acts of migration and being faced by widely different climate and food regimes. Our captive experiments revealed that neither migration nor protein limitation are required to generate the within-individual trade-off between gizzard mass and pectoral muscle mass. We speculate that the negative covariance may provide clues as to the mechanisms coordinating body remodelling in free-living birds. Although our proposed mechanism is speculative and requires empirical scrutiny, this work supports earlier calls for

greater integration of function and mechanism within studies, and in particular, the need for more explicit consideration of the evolution of mechanisms (McNamara and Houston 2009).

Acknowledgements

Funding

Electronic supplementary material

Text S1: Knot husbandry

Knots were housed in eight separate outdoor aviaries (4.0 m deep x 1.9 m wide). The roof was sloped such that the height ranged from 1.9 to 2.3 m. During experiments, each aviary contained between 14 and 17 knots in mixed flocks with *C. c. canutus* that were being kept as part of another study. Aviaries were exposed to ambient temperature and natural photoperiod, but were sheltered from the wind and rain. Aviary floors were kept wet with a constant stream of filtered sea water and a tray of running freshwater was available for drinking and bathing. In order to maintain relatively constant flock sizes and composition with respect to subspecies across the two study years, some of the birds measured in the first year of the experiment were removed for the second year of the experiment to make room for the newly captured *C. c. canutus* birds. We preferentially removed individuals for which we had missing data in the first year.

Text S2: Gizzard and pectoral muscle mass measurements

A single measurement of pectoral muscle thickness (mm) and two sets of measurements of gizzard height (cm) and gizzard width (cm) were obtained for each bird during each measurement session. Pectoral muscle mass and gizzard mass were estimated with equations obtained from regressions of similarly obtained pectoral muscle mass (grams) and fresh gizzard mass (grams) from dead birds (van den Hout et al. 2010, Dietz et al. 2013). Pectoral muscle mass was estimated using Eq. 1, and gizzard mass was estimated using Eq. 2. During the same measurement sessions, body mass (to the nearest gram) was measured using a digital balance.

Eq. 1 *pectoral muscle mass (g)= -9.58+0.28 ×pectoral muscle thickness (cm)*

Eq. 2 *gizzard mass= -1.09+3.78 ×average width (cm)×average height (cm)*

Text S3: Model implementation procedures

Multivariate analyses presented in the main text used an inverse gamma prior. However, results were quantitatively similar when using an inverse wishart prior, and were not sensitive to nu (degree of belief in the prior). We present estimates obtained from models were nu = 3. Models were run for 103,000 iterations, with a burn-in period of 3000 and a thinning interval of 100. This produced a sample of 1000 estimates. These estimates were used to calculate the most likely value for each correlation (the mode of the distribution), as well as its 95% credible interval.

Table S1. Among-individual (above diagonal) and within-individual (below diagonal) correlations estimated from a multivariate model with bird id as a random effect. Separate correlation matrices presented for each age cohort (age at capture: adult or juvenile) and each year (Year 1 or Year 2). All response variables were modelled with Gaussian errors. There was no support differences between any pairwise comparison between Year/cohort combinations in any of the estimated correlations (i.e., al 95% CIs show broad overlap), so a single covariance matrix is presented in the main text which combines both age cohorts and both years.

Adults Year 1

	Gizzard	Pectoral	Body Mass
Gizzard	--	0.38 (-0.14, 0.78)	0.34 (-0.20, 0.72)
Pectoral	-0.20 (-0.35, -0.03)	--	0.85 (0.71, 0.94)
Body Mass	0.15 (0.01, 0.31)	0.61 (0.49, 0.71)	--

Adults Year 2

	Gizzard	Pectoral	Body Mass
Gizzard	--	0.43 (-0.63, 0.93)	0.48 (-0.57, 0.94)
Pectoral	-0.16 (-0.32, 0.00)	--	0.67 (0.35, 0.86)
Body Mass	0.27 (0.08, 0.43)	0.37 (0.19, 0.53)	--

Juveniles Year 1

	Gizzard	Pectoral	Body Mass
Gizzard	--	0.64 (-0.22, 0.97)	0.65 (-0.33, 0.97)
Pectoral	-0.12 (-0.28, 0.04)	--	0.77 (0.51, 0.93)
Body Mass	0.04 (-0.11, 0.20)	0.58 (0.47, 0.69)	--

Juveniles Year 2

	Gizzard	Pectoral	Body Mass
Gizzard	--	0.71 (0.16, 0.96)	0.79 (0.33, 0.97)
Pectoral	-0.09 (-0.26, 0.10)	--	0.81 (0.62, 0.94)
Body Mass	0.32 (0.15, 0.48)	0.32 (0.14, 0.48)	--

Table S2. Slopes between body mass (g), pectoral muscle mass (g) and gizzard mass (g) estimated using reduced major axis regression (RMA) at A) the among-individual, and B) within-individual level. Values are presented as predicted change in traits along top row for a 1 g change in trait indicated in first column.

A.   Among-individual slopes

Associated with x g increase in:

1 g increase in:	Body mass (g)	Pectoral muscle mass (g)	Gizzard mass (g)
Body mass (g)	-	0.16 (0.14, 0.19)	0.05 (0.04, 0.06)
Pectoral muscle mass (g)	6.08 (5.26, 7.04)	-	0.30 (0.25, 0.37)
Gizzard mass (g)	19.95 (16.72, 23.81)	3.23 (2.68, 4.01)	-

B.   Within-individual slopes

Associated with x g increase in:

1 g increase in:	Body mass (g)	Pectoral muscle mass (g)	Gizzard mass (g)
Body mass (g)	-	0.21 (0.20, 0.22)	0.17 (0.15, 0.18)
Pectoral muscle mass (g)	4.78 (4.47, 5.12)	-	-0.79 (-0.86, -0.73)
Gizzard mass (g)	6.04 (5.57, 6.54)	-1.26 (-1.37, -1.16)	-

WITHIN-INDIVIDUAL CANALIZATION CONTRIBUTES TO AGE-RELATED INCREASES IN TRAIT REPEATABILITY: A LONGITUDINAL EXPERIMENT IN RED KNOTS

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Age-related increases in the repeatable expression of labile phenotypic traits are often assumed to arise from an increase in among-individual variance due to differences in developmental plasticity or by means of state-behaviour feedbacks. However, age-related increases in repeatability could also arise from a decrease in within-individual variance as a result of stabilizing trait expression, i.e. canalization. Here we describe age-related changes in within- and among-individual variance components in two correlated traits, gizzard mass and exploration behavior, in a medium-sized shorebird, the red knot (*Calidris canutus*).

Increased repeatability of gizzard mass came about due to an increase in among-individual variance, unrelated to differences in developmental plasticity, together with decreases in within-individual variance, consistent with canalization. We also found canalization of exploration, but no age-related increase in overall repeatability, which suggests that showing predictable expression of exploration behaviour may be advantageous from a very young age onward. Contrasts between juveniles and adults in the first year after their capture provide support for the idea that environmental conditions play a key role in generating among-individual variation in both gizzard mass and exploration behavior. Our study shows that stabilization of traits occurs under constant conditions: with increased exposure to predictable cues, individuals may become more certain in their assessment of the environment allowing traits to become canalized.

Introduction

Individuals often differ consistently from one another in suites of behavioral, physiological, and morphological traits (Sih et al. 2004, Bell et al. 2009, Réale et al. 2010*a*, Dall et al. 2012, Carere and Maestripieri 2013). Although inter-individual variability was traditionally viewed as merely the substrate for natural selection, evidence is accumulating that among-individual variation has greater ecological and evolutionary implications (Bolnick et al. 2003, Sih et al. 2012, Wolf and Weissing 2012). It is increasingly recognized that such intraspecific variation may be adaptive (Wilson and Yoshimura 1994, Bolnick et al. 2003, Dall et al. 2004, Sih et al. 2004, Réale et al. 2007).

Individuals of the same population may differ in dispersal behavior (Cote et al. 2010*a*, 2010*b*), foraging behavior, resource use (Svanbäck and Bolnick 2007, Toscano et al. 2016, Sheppard et al. 2018), and aggression (Bell et al. 2009). In some cases, these among-individual differences in behavior are associated with differences in reproduction and survival (Wilson 1998, Smith and Blumstein 2008, Réale et al. 2010*b*). Variation between individuals can lead to non-random distributions of individuals, an increase in the range of resources that can be exploited, and higher resilience to change for populations as a whole (Bolnick et al. 2003, Wolf and Weissing 2010, Sih et al. 2012). Therefore, not surprisingly, a significant amount of work has aimed to address the causes of among-individual variation (Wolf et al. 2007, Dingemanse and Wolf 2010).

The ontogeny of repeatable among-individual variation, a potentially core process underlying individual development, has received markedly less attention (but see Sinn et al. 2008, Bell et al. 2009, Biro and Stamps 2015, Polverino et al. 2016). Although among individual variance can in theory either increase or decrease with age, the majority of empirical studies have reported age-related increases in repeatable

among-individual variation (Table 1). However, these studies often fail to evaluate whether these changes are driven by changes in the amount of among-individual variation, within-individual variation, or both (Table 1).

Theoretical considerations of the development of trait repeatability have focused on processes that affect the amount of variation among individuals (Sih et al. 2015, Fisher et al. 2018, Stamps and Frankenhuis 2016). One obvious explanation for age-related increases in among-individual trait expression is that it reflects among-individual differences in the conditions experienced during development (West-Eberhard 2003). However, among-individual variation can also arise when individuals are reared under near-identical conditions (Crabbe et al. 1999, Brust et al. 2015, Bierbach et al. 2017). This could be due to (epi-) genetic variation among individuals (Dall et al. 2012) or to individual differences in developmental plasticity (i.e., the effect of environment on phenotypic differences) (West-Eberhard 1989, 2003, Stamps and Krishnan 2014a, Stamps and Frankenhuis 2016).

Among-individual variance can also increase over time through positive-feedbacks between two traits (Sih et al. 2015). For example, foraging boldness (i.e., willingness to forage in the presence of predators), may allow individuals to acquire more resources and grow relatively more quickly compared to individuals that do not forage in the presence of predators (Luttbeg and Sih 2010). If, at the same time, being larger confers some safety advantage (e.g., because predators are gape limited and large prey are less accessible), then being larger will also favor higher boldness (Luttbeg and Sih 2010). The reciprocal effects of boldness on body size, and body size on boldness, mean that initially very small differences between individuals can increase over time (Sih et al. 2015).

Though less often considered, increased repeatability during development may also result from decreasing within-individual variance, or canalization (Waddington 1942). A trait is considered canalized if phenotypic expression remains invariable under mildly differing developmental conditions. The term canalization was originally used to refer to the evolution of developmental stability (Waddington 1942). According to Waddington's (1942) metaphor of canalization, the individual development of traits can be visualized as the movement of balls rolling down alternative valleys in a landscape that has been shaped by natural selection. Environmental effects can either be implemented either as modifications to the width and depth of a single valley (Boonekamp et al. 2018) or as switches between alternative valleys (Waddington 1942). More recently the term canalization has been applied to refer to the reduction in residual phenotypic variance at the within-individual level (Westneat et al. 2015). A reduction in within-individual variance (i.e., residual variance) can occur if phenotypic variation decreases in the course of development (e.g., Stamps and Krishnan 2014*b*, 2017, Westneat et al. 2015, Stamps and Frankenhuis 2016). Following Waddington's metaphor, this is analogous to the valleys in the phenotypic landscape to deepen and/or narrow over time, producing more rigid and predictable trait expression across ontogeny (Boonekamp et al. 2018).

Thus, age-related increases in trait repeatability under identical conditions can be the outcome of at least three distinct developmental processes. Two of these affect the degree of among-individual variance (i.e. differences in developmental plasticity and state-behavior feedbacks) and one affects the degree of within-individual variance (within-individual canalization) (See Table 2 and Figure A1 for a matrix of predictions). Recognizing that many organisms are only sensitive to environmental cues during particular stages of ontogeny (Bateson 1979, Fawcett and Frankenhuis 2015, Panchanathan and Frankenhuis 2016), systematic investigation of the development of trait expression

requires longitudinal studies of development.

We studied the development of two ecologically important phenotypic traits in a migratory shorebird: gizzard mass (Piersma et al. 2003, van Gils et al. 2003, 2005) and exploration behavior (Bijleveld et al. 2014, 2016, Oudman et al. 2016). In a longitudinal study spanning two consecutive years, we measured individual changes in gizzard mass and exploration behavior in red knots *(Calidris canutus;* hereafter called 'knot'). During the non-breeding season, knots forage on a diet of hard-shelled prey, primarily mollusks, that they crush in their muscular gizzards (Zwarts and Blomert 1992, Piersma et al. 1993b, Battley and Piersma 2005a), and gizzard mass is therefore a key trait (van Gils et al. 2005b). Further, exploration behavior scored in standardized behavioral assays (see Methods) has been shown to be correlated with large-scale (100s of km) patterns of space use in the wild (Bijleveld et al. 2014). Notably, exploration and gizzard mass in free-living knots co-vary at the among individual level; individuals with large gizzards at the time of capture have lower exploration scores than individuals with small gizzards (Bijleveld et al. 2014, 2016). Previous laboratory experiments have shown that both gizzard mass and exploration behavior exhibit repeatable among-individiual variation in knots (≥ 2 calendar year; see "Material and Methods" for explanation; Bijleveld et al. 2014, Mathot et al. 2017). However, experimental manipulations of gizzard mass produced no changes in exploration behavior (Bijleveld et al. 2014). These series of observations led to the speculation that the among-individual variation in gizzard mass and exploration behavior could be the result of state-behavior feedbacks between searching behavior and digestive quality of food during a limited window in early ontogeny (Bijleveld et al. 2014). Here we describe the development of age-related difference in trait repeatability in the light of three non-exclusive developmental processes to elucidate the developmental origin of among-individual variation in trait expression.

Table 1. A non-exhaustive review of papers reporting age-related changes in trait repeatability.

Species	Age group	Trait	Age-related effect	R/VC	Ref
Insects					
Red flour beetle (*Tribolium castaneum*)	Sub-adult → Young Adult → Adults	B: Movement	–	R	Wexler et al. 2016
		B: Edge-preference	–	R	
Wild cricket (*Gryllus campestris*)	Juveniles → Adults	B: Flight initiation distance	–	VC	Niemelä and Dingemanse 2017
Speckled wood butterfly (*Pararge aegeria*)	Juvenile → Adult	B: Activity[1]	–[2]	VC	Kaiser et al. 2018
Arachnids					
Desert funnel-web spider (*Agelenopsis lisa*)	Juvenile → penultimate molt → sexually mature	B: Foraging	↑	R	Bosco et al. 2017
		B: Exploration	↑/↓[3]	R	
		B: Neophobia	– (M), ↑ (F)	R	
		B: Boldness	↑ (M), – (F)	R	
Basal tarantula (*Brachypelma smithi*)	Juvenile (1-year old) → immature (2-year old)	B: Boldness	–[3]	R	Bengston et al. 2014
		B: Latency to attack	–[3]	R	
		B: Conspecific tolerance	↑[3]	R	
		B: Neophobia	–[3]	R	
Fish					
Eastern mosquitofish (*Gambusia holbrooki*)	Juvenile → Sub-adult → Adult	B: Distance moved	↑	R	Polverino et al. 2016
		B: Freezing time	↑	R	
		B: Hiding time	↑	R	
		B: Latency to emerge	↑	R	

Table 1. Continued

Species	Age group	Trait	Age-related effect	R/VC	Ref
Fish					
Amazon molly (*Poecilia formosa*)	Hatchling → Juvenile	B: Movement	↑	VC	Bierbach et al. 2017
White shark (*Carcharodon carcharis*)	Sub-adult → Adult	B: Prey choice	↑	VC	Kim et al. 2012
Crayfish (*Cherax destructor*)	0 → 4 months	P: Growth rate	↑	R	Biro et al. 2014
		B: Boldness	↑		
Reptiles					
Western fence lizard (*Sceloporus occidentalis*)	Hatchling → Juvenile → Sub-adult	B: Locomotion performance	–	R	Van Berkum et al. 1989
		M: Body size	–	R	
Gecko (*Lepidodactylus lugubris*)	Juveniles → Adult	B: Exploration	–[2]	VC	Sakai 2018
		B: Boldness	–[2]	VC	
Birds					
Zebra finch (*Taeniopygia guttata*)	Sub-adult → Young adult → Adult	B: Fearlessness	–	R	Wuerz and Krüger 2015
		B: Exploration	–	R	
		B: Activity	–	R	
		B: Aggression	–	R	
		B: Boldness	–	R	
House sparrow (*Passer domesticus*)	Nestling → Adult	P: Corticosterone levels	–	R	Lendvai et al. 2015

Table 1. Continued

Species	Age group	Trait	Age-related effect	R/VC	Ref
Mammals					
Yellow bellied marmot (*Marmot flaviventris*)	Juvenile →/ Yearling →/ Adult	B: Boldness	↑/↓[4]	R	Petelle et al. 2013
		B: Docility	↑	R	
Eurasian harvest mice (*Micromys minutus*)	Juvenile → Adult	B: Exploration	–	R	Schuster et al. 2017
		B: Activity	↑	R	
		B: Boldness	–/↓[5]	R	
		C: Spatial Recognition	↑	R	
European roe deer (*Capreolus capreolus*)	Juvenile/Adult	B: Docility	–	R	Debeffe et al. 2015

Note. - In each study age groups were compared using either a longitudinal approach (→; i.e. measuring the same individual over different life stages) or a cross-sectional approach (/ ; i.e. comparing individuals belonging to different life stages) or a combination of both approaches. The type of trait is indicated as B (behavioral), M (morphological), P (physiological) or C (cognitive), with a brief description. Age related effects are summarized as increasing (↑), no change (–) or decreasing repeatability (↓). In situations that effect differed between sexes this is indicated with M (males) and F (females). The column labeled R/VC indicates whether only changes in repeatability were considered (R), or whether changes in within- and among-individual variance components were assessed (or provided) separately (VC).

[1]Note that other behaviors were also scored in this study, but the only common assay across age cohorts was activity.

[2]Did not formally compare the repeatability across the age classes, but point estimates were presented and could be compared.

[3]Repeatability increased from juvenile to penultimate molt, decreased from penultimate molt to mature adult

[4]Juvenile and adult marmots showed no repeatability in boldness but yearlings did.

[5]Boldness is repeatable in juveniles and in adult males but not in females. This implies an age-related decrease in repeatability for females. But this is not explicitly discussed in the paper.

Table 2. Predicted alternative processes that may generate age-related increases in trait repeatability.

Process	Among-individual variance	Within-individual variance	Within-individual trait covariance
Individual differences in developmental plasticity	↑	–	N[1]
State-behavior feedbacks	↑[2]	–	Y[3]
Within-individual canalization	–	↓	N

Note. - Predicted age-related changes in among- and within-individual variance (↑ = increase, ↓ = decrease, - = no prediction), and the presence of within-individual trait covariances (Y = Yes, N = No) for three alternative processes that may generate age-related increases in trait repeatability. Predicted age-related changes in among-individual variance and within-individual variance apply both within-individuals over time, as well as across age categories (e.g., between juveniles versus adults).

[1]Within-individual trait covariance is not explicitly predicted from individual differences in developmental plasticity. However, when developmental plasticity of two traits covaries, this could generate within-individual trait covariance.

[2]Our experimental protocol interrupts potential state-behavior feedbacks (see Methods). Thus, we would not predict to generate increased among-individual variance within-individuals over time. However, we would expect to find lower among-individual variance in juveniles than in adults in year-1, as knots captured as adults would have experienced state-behavior feedbacks prior to capture.

[3]Within-individual trait covariance present during ontogeny, absent after ontogeny.

Material and methods

Study species and housing conditions

The knots (*islandica* subspecies; Piersma 2007) used in this study were captured with mist nets at two different high tide roosts in the Dutch Wadden Sea; Schiermonnikoog (53.29°N, 6.15°E) (n=53) and Griend (53.15°N, 5.16°E) (n=31) between 20 August and 20 October 2015. Birds were aged based on plumage characteristics as either juvenile birds (< 6 months), second calendar year birds (between 6 and 18 months), or older (i.e., adult birds, > 18 months) (Prater et al. 1977). Only juveniles and adults were selected for the study (N = 44 juveniles, N = 46 adults). We collected a small blood sample (< 75 µl) for molecular sexing (van der Velde et al. 2017). For simplicity, we refer to the birds caught as first year birds as "juveniles" throughout the paper, despite the fact that they changed from being juvenile to second calendar year to adults in the course of this two-year-long study.

Birds were housed in outdoor aviaries (4.0 m deep, 1.9 m wide, and 2.3 m high at one end, sloping down to a height of 1.9 across the depth of the aviary) at the Experimental Shorebird Facility of the NIOZ Royal Netherlands Institute for Sea Research on the island of Texel, The Netherlands (53°00'N, 04°47'E). The aviaries had smoothly coated concrete floors that were constantly irrigated with running seawater. The back of each aviary had a basin with sand collected from the Wadden Sea and running seawater. Outside of experiments, birds had *ad libitum* access to Trout food pellets (Produits Trouw, Vervins, France) and a continuous source of fresh water for drinking and bathing in a separate tray. Every week, while the aviary floors were cleaned and disinfected with chlorine, the birds were weighed, their molt and plumage status scored and their bodies, especially their feet, checked for small wounds and *Staphylococcus* infection (Milot et al. 2014).

The focal birds (*islandica* subspecies) were kept together with knots of the *canutus* subspecies in mixed flocks (14-17 knots per aviary, randomized with stratification based on age and subspecies). Flock composition was largely constant throughout the first year, but before the start of the second year of experiments new birds of the *canutus* subspecies were caught (N = 22). Thus, to maintain constant flock sizes across the two study years, 24 *islandica* knots were released between year-1 and year-2 of the experiment.

Diet manipulations

To prevent circannual endogenous rhythms from unduly affecting our measurements (Battley and Piersma 2005a, Karagicheva et al. 2016), experiments were only carried out over two non-breeding periods, from late October 2015 to early April 2016 in year-1, and from early October 2016 to mid-March 2017 in year-2. During the experimental period, birds were fed *ad libitum* diets of either high or low digestive quality. The high digestive quality food (HQ) consisted of Trout food pellets and the low digestive quality (LQ) food was thawed mud snails, *Peringia ulvae*. Previous work has shown that these two food types induce approximately two-fold variation in gizzard mass (Vézina et al. 2011, Mathot et al. 2017). We used a crossover design: birds in half of the aviaries (n=4) were fed HQ food first, while the other half received LQ food first. Previous studies showed that knots can fully adjust their gizzard mass to a new food type within approximately one week (Dekinga et al. 2001), but we allowed three weeks of acclimatization to the new diet to ensure that the general condition of the birds would be stable and equal between diets (Bijleveld et al. 2014, Mathot et al. 2017).

These three weeks of diet manipulation were followed by two weeks of behavioral observation, during which time the knots remained on the same *ad libitum* diet. When all behavioral observations were completed, a new replicate of diet manipulations commenced; aviaries

previously assigned the HQ food treatment became LQ aviaries and *vice versa.* Four diet treatments were carried out per bird during each of the two experimental years. To prevent for systematic differences between the knots as a result of the order of testing, we randomized the sequence with which we tested individuals in each behavioral test. On average 43 days (ranging between 21-65 days) elapsed between successive behavioral tests in year-1 and 40 days (ranging between 24-57 days) between successive tests in year-2.

Gizzard mass measurements

After each diet treatment, and before behavioral observations, the gizzard mass of all birds was measured using ultrasonography (Dekinga et al. 2001, Dietz et al. 1999a). To standardize the measurements, birds were deprived of food for at least one hour prior to measurement to ensure an empty gizzard. Subsequently birds were selected in a haphazard order for measurement. The observer was blind to the age and diet treatment of the birds. Gizzard measurements were done following a standardized procedure developed by AD, see Mathot et al. (2017) for a detailed description of the method.

Exploration behavior

The exploration behavior of individual birds was quantified in an arena that was novel for the birds during first exposure. Studies on exploration traditionally focus on individual movements after introduction to a novel environment (Verbeek et al. 1994, Réale et al. 2007). Studies that assess repeatability of exploration typically re-use the same test arena for subsequent tests (e.g., Dingemanse et al. 2002, 2012, Minderman et al. 2010, Bijleveld et al. 2014, McCowan et al. 2015, Wuerz and Krüger 2015, Dubuc-Messier et al. 2017). Therefore, a decline in novelty with repeated exposure is an inherent feature of studies estimating repeatability in exploration. The exploration arena used here was identical to the one used in Bijleveld et al. (2014). It measured 7 x 7 m and was filled with a layer of 30 cm seawater and five

1m x 1m trays filled with wet sand (Figure A2).

Birds were caught from their holding aviaries two hours prior to their randomly assigned observation time and kept individually in holding crates in a semi-dark and quiet room. Birds were food-deprived during these two hours to standardize hunger levels between birds. Immediately prior to the test, each bird was moved to a small aviary adjacent to the arena. After five minutes of acclimatization, the door between the aviary and the arena was opened by means of a remote pulley system and the bird was gently herded into the arena. Exploration trials lasted 30 minutes, during which time the behavior of the bird was scored live through one-way-glass using the behavioral observation software, JWatcher (http://www.jwatcher.ucla.edu/) and recorded (GoPro HERO+ LCD) for future reference. The observations were done by five different observers (three observers in year-1, and four observers in year-2, one common observer across both years) who were randomly distributed over the trials and blind to the treatment and age of the birds being tested. Behaviors recorded were: flying, walking on patches, searching for food, preening, resting, vigilance, out of sight, or 'other'. After the trial ended, the bird was returned to its holding aviary.

Using the protocol developed by Bijleveld et al. (2014) for the same subspecies of red knot, exploration behavior was scored as the fraction of time spent searching or walking on patches. Proportions were logit transformed to meet normality assumptions (Warton and Hui, 2011). This measure of exploration may not be assaying information gathering by individuals in a broad sense, but more specifically information gathering related to the distribution of food.

All experiments described in this paper complied with Dutch law and regulation and were carried out under protocol number AVD802002016740.

Data selection and statistical analyses

We obtained 569 complete observations of exploration behavior and gizzard mass (N_{adult} = 283, $N_{juvenile}$ = 286). We excluded replicates during which the exploration experiments were disturbed (n = 2), when birds swam in the arena for more than 10 consecutive min (n = 11) because of welfare concerns for the bird (i.e., the risk of drowning or inability to thermoregulate with wet feathers), or when the diet manipulation was unsuccessful (n = 14). Unsuccessful diet manipulations occurred when knots failed to switch to the experimentally determined diet (as evidenced by significant body mass loss), or when knots had to be removed from the experiment to be treated for *Staphylococcus* infection.

We constructed univariate models for gizzard mass and exploration behavior to study the development of age-related difference in trait repeatability. To be able to compare both age groups between year-1 and year-2, as well as to contrast adults and juveniles, we constructed separate models for each age group in each year (i.e., 4 models per trait; juveniles year-1, juveniles year-2, adults year-1, adults year-2). Because we were explicitly interested in age- and year-specific estimates for both among- and within-individual variance components, we included a random intercept for individual ID.

Although contrasts between the among- and within-individual variance components for each age-cohort and year combination could have been carried out in a single analysis by modelling heterogeneous residual errors, such analyses have very low statistical power (Cleasby and Nakagawa 2011). Therefore, we split the data in 4 bins and estimated the variance components for each trait per age group and year. To be able to correctly calculate within-individual variance that was unrelated to diet we used two measures of gizzard mass and exploration on each diet per year following Araya-Ajoy et al. (2015). We obtained complete

data records (i.e., two measures of gizzard mass and exploration on each diets in each year ($N_{replicates\_per\_bird}$ = 8) for a total of 58 birds (30 "adults" and 28 "juveniles" ($N_{adult\_measurements}$ = 240, $N_{juvenile\_measurement}$ = 224). This data can be found in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.dn28cn6 (Kok et al. 2019b). We restricted our analyses to these birds, as any changes in variance components from year-1 to year-2 necessarily reflected within-individual, age- (or time in captivity-) related changes in variance components, as opposed to changes resulting from comparing different cohorts of birds. However, our data selection criteria did not affect the estimates of either the fixed effects or the variance components (results not shown).

We modeled gizzard mass and logit exploration as a function of sex (2-level factor: M or F), diet (2-level factor: LQ or HQ), replicate (continuous factor: range 1-4) and the interaction between diet and replicate. The addition of replicate in the model allowed us to test for changes in the response variables over time. The interaction term between diet and replicate allowed for a comparison of diet-related differences in the effect of replicate. In the results section, we focus on the effects of diet, replicate (time), and their interaction, on gizzard mass and exploration. We did, however, also include a fixed effect for sex to control for potential differences due to structural size differences between the sexes, since female knots are larger than males (Tomkovich 1992), but we will not discuss this any further in the results. For the models of exploration behavior, we fitted an additional random intercept for observer ID, to control for potential among-observer differences in behavioral scoring that would otherwise introduce additional residual variance. Because the observer was blind to the age group and diet treatment of each experimental bird, and because birds were randomly assigned to each observer, observer effects are not biologically meaningful, and are not relevant for the hypotheses being tested. They are presented in Table 3 for completeness, but are not discussed further. Models were built using the *lmer* function

from the *lme4 package* (Bates et al. 2015) in the R (v3.4.3) statistical environment (R Core Development Team 2017).

We report adjusted repeatabilities (i.e. after correcting for the fixed effects in the model) that were calculated following Nakagawa and Schielzeth (2010, 2013). To study the age-dependent changes in repeatabilities, we first compare the changes in repeatability of gizzard mass and exploration for juveniles and adults between year-1 and year-2 in a longitudinal analyses. Subsequently we report a cross-sectional comparisons (e.g., comparing juveniles in year-1 with adults in year-1 as well as juveniles in year-2 with adults in year-2) to separate age-dependent effects from effects resulting from free-ranging experience or time in captivity. In all cases, we report how both within- (i.e., residual) and among-individual variance components contributed to the overall repeatability (Cleasby and Nakagawa 2011). In cases where we found a value of zero for the among-individual variance, we verified that this was not a false negative result (e.g., singularity due to model overfitting), by rerunning the model with bird ID fitted as a fixed effect rather than a random effect and evaluating its effect size. We found no evidence that any of our estimates of zero among-individual variance were due to model overfitting (results not shown).

We used the *sim* function of the *arm* package (Gelman and Su 2016), to simulate values of the posterior distribution of the model parameters (Gelman and Hill 2007). 95% credible intervals (CIs) around the mean (β) were extracted based on 1000 simulations using the *MCMCglmm* package (Hadfield 2010). The 95% CI indicates a *margin of error* in terms of a range of plausible values for β. With this CI we indicate that we are 95% confident that our CI includes the actual effect size (Cumming and Finch 2005). To evaluate the differences between means and CIs of the fixed effects, as well as the variance components and the repeatability estimates, we followed Cumming and Finch (2005).

Independent 95% CIs were deemed to indicate significant differences between averages when they did not overlap. This corresponds to a traditional p value < 0.006. In cases that the 95% CIs did overlap, we used the proportion of overlap of the 95% CIs (the overlap between the two independent CIs divided by the average CI) to calculate the exact probability (p) that two CIs overlap (Cumming 2009) and discuss the level of support for a given effect as a continuous, rather than binary, characteristic.

Results

Changes in mean trait values

In each age group in both years, knots developed heavier gizzards on the LQ diet treatment than on the HQ diet treatment (95% CIs for fixed effect diet do not overlap with 0, except for a 0.15 proportion of overlap in juveniles in year-2, Table 4). Within each year, diet effects were similar between juveniles and adults, indicating similar gizzard mass plasticity across age cohorts. With time, the relatively light gizzard mass as a result of the HQ diet increased for both juveniles and adults (in year-2 the 95% CIs for replicate do not overlap with 0, Table 4). The negative interaction effect between diet and replicate found for adults in year-1 showed a decrease in gizzard masses on the LQ diet treatment across replicates (Table 4). These contrasting effects of replicate on HQ versus LQ diet meant that the overall diet effect decreased across replicates (Figure 1).

Exploration did not differ significantly between years or across age groups (all 95% CIs overlap), but exploration behavior decreased across replicates in adults in year-1 (95% CI for replicate does not overlap with 0, Table 3). Contrary to expectation, we found no effect of diet on exploration in any of the age groups (all 95% CI overlap with 0, Table 3, Figure 2).
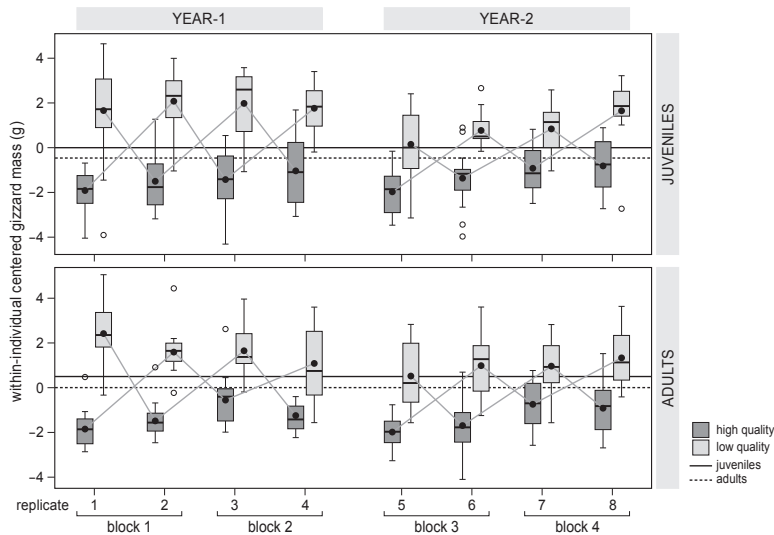
Figure 1. Gizzard mass as a function of manipulated diet and time. Boxes represent the median, quartiles and interquartile outliers in within-individual centered gizzard mass (g) for juveniles (top) and adults (bottom). Gizzard mass was centered within individual by subtracting the individuals mean from each measurement (n=8). Dark grey indicate birds on high quality food (pellets) and the light grey indicate birds on low quality food (mudsnails). Means (black circles) are connected with grey lines for each group of individuals receiving the similar treatment order in the crossover design. The horizontal black lines represent the mean gizzard mass for juveniles (continuous line) and for adults (interrupted line). Measurement taken during the non-breeding season in year-1 (i.e. replicate 1-4) are separated from measurements taken during the non-breeding season in year-2 (i.e. replicate 5-8) by a breeding summer when no measurements were taken.
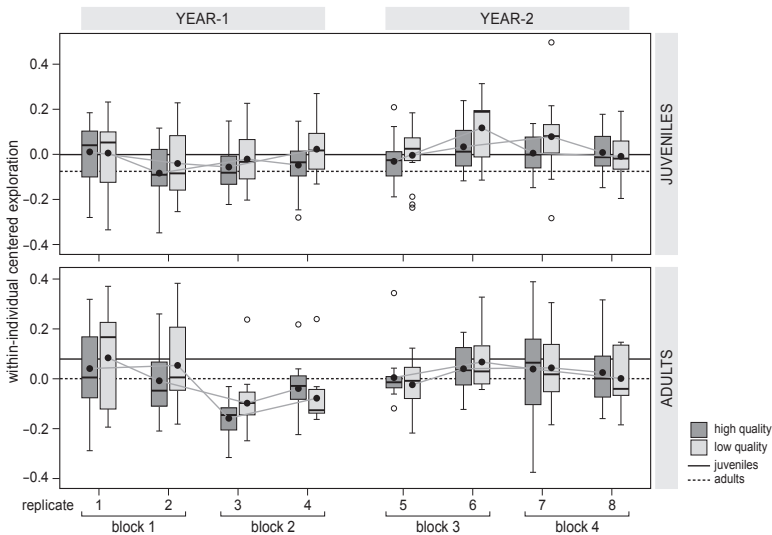
Figure 2. Exploration behavior scores as a function of manipulated diet and time.
Boxes represent the median, quartiles and interquartile outliers in within-individual centered score for exploration behavior for juveniles (top) and adults (bottom). Exploration was centered within individual by subtracting the individuals mean from each measurement (n=8). Dark grey indicate birds on high quality food (pellets) and the light grey indicate birds on low quality food (mudsnails). Means (black circles) are connected with grey lines for each group of individuals receiving the similar treatment order in the crossover design. The horizontal black lines represent the mean exploration behavior for juveniles (continuous line) and for adults (interrupted line). Measurement taken during the non-breeding season in year-1 (i.e. replicate 1-4) are separated from measurements taken during the non-breeding season in year-2 (i.e. replicate 5-8) by a breeding summer when no measurements were taken.

Age-dependent changes in trait repeatability

Juvenile knots showed no repeatability in gizzard mass in year-1 (r and 95% CI centered on 0) and a small but significant repeatability in year-2 (95% CI does not overlap with 0, Table 4, Figure 3). In contrast, juvenile knots already showed significant repeatability in exploration behavior in year-1 (95% CI does not overlap with 0) and there was no support for change in the repeatability between year-1 and year-2 (p = 0.29, Table 3, Figure 3).

In adults we observed significant repeatability in gizzard mass in both year-1 and year-2 (95% CIs do not overlap with 0). However, the repeatability in year-2 was significantly lower (95% CIs do not overlap, Table 4, Figure 3). For exploration behavior in adults we found significant (95% CIs do not overlap with 0), and near identical repeatabilities in year-1 and year-2 (proportion overlap between years: p = 0.89, Table 3, Figure 3).

When comparing gizzard mass repeatabilities between age groups, repeatability was lower in juveniles than in adults in year-1 (95% CIs do not overlap). In year-2, both repeatability estimates were very small but juvenile repeatability was higher when compared to adults (p < 0.01, Table 4, Figure 3). For exploration behavior, we found no differences in repeatability between adults and juveniles in year-1 (p = 0.54) nor in year-2 (p = 0.90, Table 3, Figure 3).

Changes in among- and within-individual variance components
In juveniles, the increase in gizzard mass repeatability between year-1 and year-2 was the result of an increase in among-individual variance (95% CIs do not overlap) together with a decrease in within-individual variance of 32% between year-1 and year-2 (p = 0.03, Table 4, Figure 3). For exploration behavior we found no significant change in repeatability between year-1 and year-2. However, in the absence of a change in among-individual variance in juveniles (p = 0.90, Table 3, Figure 3), a significant decrease in within-individual variance (p < 0.01) led to a (non-significant) increase in repeatability.
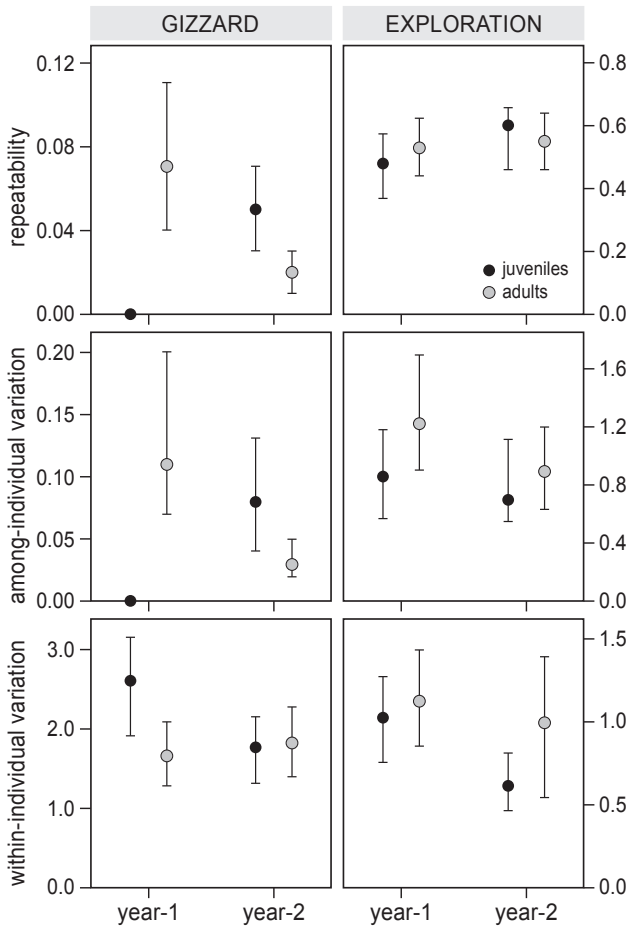
Figure 3. Development of variance components of gizzard mass and exploration behavior. Adjusted repeatabilities (top), among-individual variation (middle) and within-individual variation (bottom) for gizzard mass (left) and exploration behavior (right) for juveniles (black) and adults (grey) in year-1 and year-2. Dots and bars represent β + 95 % CI for each age group per year.

In adults, the significant decrease of gizzard mass repeatability between year-1 and year-2 was the result of a decrease in among-individual variation (95% CIs do not overlap), while the within-individual variance did not change (p = 0.90, Table 4, Figure 3). We found no change in repeatability in exploration behavior for adults from year-1 to year-2. However, when considering the changes in among- and within-individual variance components separately, we found a decrease in the within-individual variance (p = 0.01) together with a non-significant concomitant decrease in among-individual variance (p = 0.15, Table 3, Figure 3)

The age-related difference in gizzard mass repeatability between juveniles and adults in year-1 was due to lower among-individual variance and higher within-individual variance in juveniles compared with adults (among-individual variance; 95% CIs do not overlap, within-individual variance; p = 0.01, Table 4, Figure 3). In year-2, the small but significantly higher repeatability in gizzard mass of juveniles compared to adults was the result of higher among-individual variance in juveniles than in adults (p = 0.01). The within-individual variance did not differ between juveniles and adults (p = 0.85, Table 4, Figure 3).

As for repeatability, the within- (p = 0.73) and among-individual variance (p = 0.11) in exploration behavior did not differ between juveniles and adults in year-1 (Table 3, Figure 3). However, the limited overlap in 95% CIs in among-individual variance in adults and juveniles suggests that adults showed higher among-individual variance than juveniles in year-1 (Table 3, Figure 3). In year-2, there was no difference between adults and juveniles in either the within- (p = 0.63) or among-individual (p= 0.92) variance in exploration behavior (Table 3, Figure 3).

Table 3. Sources of variation in logit transformed exploration behavior.

	Juveniles		Adults	
	Year-1	Year-2	Year-1	Year-2
Fixed effects	β ± 95% CI	β ± 95% CI	β ± 95% CI	β ± 95% CI
Intercept[1]	-0.92 (-1.86,0.04)	-1.67 (-2.89,-0.27)	-0.98 (-1.87,0.35)	-1.37 (-2.99,0.18)
Diet (LQ)	-0.08 (-1.18,0.76)	0.30 (-1.63,2.10)	0.39 (-0.55,1.44)	0.68 (-1.54,2.32)
Sex (M)	0.14 (-0.57,0.97)	0.56 (-0.20,1.27)	-0.14 (-1.11,0.75)	-0.61 (-1.19,0.35)
Replicate	-0.14 (-0.40,0.14)	0.00 (-0.12,0.26)	-0.34 (-0.60,-0.09)*	0.12 (-0.13,0.26)
Diet (LQ):Replicate	0.14 (-0.21,0.50)	-0.01 (-0.30,0.27)	-0.20 (-0.53,0.20)	-0.07 (-0.37,0.22)
Random effects	σ ± 95% CI	σ ± 95% CI	σ ± 95% CI	σ ± 95% CI
Bird ID	0.86 (0.57,1.18)	0.70 (0.55,1.11)	1.23 (0.91,1.70)	0.90 (0.63,1.20)
Observer	0.02 (0.00,0.13)	0.00 (0.00,0.00)	0.15 (0.01,0.13)	0.00 (0.00,0.00)
Residual variance	1.02 (0.75,1.26)	0.61 (0.46,0.80)	1.12 (0.84,1.42)	0.69 (0.54,0.92)
Repeatability	r ± 95% CI	r ± 95% CI	r ± 95% CI	r ± 95% CI
Bird ID	0.48 (0.37,0.57)	0.60 (0.46,0.66)	0.53 (0.44,0.62)	0.55 (0.46,0.64)

Note. – Exploration behaviour is defined as the fraction of time searching.

[1] Intercept estimated for females (F) on high quality (HQ diet) during their first replicate.

* p < 0.006 (i.e., 95% CI does not overlap zero)

Table 4. Sources of variation gizzard mass (g).

	Juveniles		Adults	
	Year-1	Year-2	Year-1	Year-2
Fixed effects	β ± 95% CI	β ± 95% CI	β ± 95% CI	β ± 95% CI
Intercept[1]	4.50 (3.22,5.42)	2.42 (0.81,5.06)	4.04 (3.12,4.91)	2.03 (-0.20,3.85)
Diet (LQ)	4.13 (2.84,5.72)*	1.97 (-0.88,4.96)	4.86 (3.78,6.06)*	4.23 (1.44,7.21)*
Sex (M)	-0.56 (-1.16,-0.07)*	-1.02 (-1.53,-0.56)*	-0.64 (-1.20,-0.13)*	-0.90 (-1.31,-0.31)*
Replicate	0.38 (-0.06,0.70)	0.46 (0.10,0.73)*	0.26 (0.00,0.58)	0.44 (0.16,0.76)*
Diet (LQ):Replicate	-0.29 (-0.88,0.17)	0.05 (-0.44,0.44)	-0.90 (-1.19,-0.34)*	-0.44 (-0.74,0.13)
Random effects	σ ± 95% CI	σ ± 95% CI	σ ± 95% CI	σ ± 95% CI
Bird ID	0.00 (0.00,0.00)	0.08 (0.04,0.13)	0.11 (0.07,0.20)	0.03 (0.02,0.05)
Residual	2.60 (1.91,3.14)	1.77 (1.30,2.14)	1.67 (1.27,2.08)	1.82 (1.38,2.27)
Repeatability	r ± 95% CI	r ± 95% CI	r ± 95% CI	r ± 95% CI
Bird ID	0.00 (0.00,0.00)	0.05 (0.03,0.07)	0.07 (0.04,0.11)	0.02 (0.01,0.03)

Note. - [1] Intercept estimated for females (F) on high quality (HQ diet) during their first replicate.

* p < 0.006 (i.e., 95% CI does not overlap zero)

Discussion

In this study, we evaluated support for three non-exclusive developmental processes that may underlie age-related changes in repeatable trait expression in knots (individual difference in phenotypic plasticity, state-behavior feedbacks, and within-individual canalization, Table 2). From year-1 to year-2 gizzard mass repeatability increased in juveniles and decreased in adults. This increase in gizzard mass repeatability in juveniles was the result of an increase in among-individual variance and a decrease in within-individual variance. In adults the decrease in repeatability was due to a decrease in among-individual variance alone. Initially (in year-1), juveniles showed lower among-individual variance and higher within-individual variance in gizzard mass than adults. In year-2 within-individual variance in juveniles declined to levels similar to older individuals (i.e. adults in year-1 and year-2). We found no linear age-related changes in among-individual variance in gizzard mass. Although exploration repeatability did not differ between age groups and years, we found a significant decrease in within-individual variance for both juveniles and adults between year-1 and year-2.

The observed age-related differences in gizzard mass repeatability resulted from changes in both the among- and within-individual variance components (Table 4). We can exclude the possibility that feedbacks led to an increase in gizzard mass repeatability, because state-behavior feedbacks would have presented themselves as within-individual correlations between gizzard mass and exploration (Luttbeg and Sih 2010, Sih et al. 2015), for which we found no support (Table 2 process 2, Table A1). We considered the possibility that the observed increase in among individual difference in gizzard mass in juveniles between year-1 and year-2 came about as a result of individual differences in developmental plasticity (Table 2 process 1). However, since we found a decrease in among-individual variance in

gizzard mass in adults between year-1 and year-2, we do not interpret these, apparently reversible, changes in among-individual variance in gizzard mass as the outcome of individual differences in developmental plasticity (Table 2 process 1; Figure A1A; West-Eberhard 1989, 2003). A reduction of within-individual variance contributed to increased repeatability in gizzard mass in juveniles between year-1 and year-2. In year-1, the within-individual variance in gizzard mass was higher in juveniles than in adults. However, between year-1 and year-2 within-individual variance in juveniles decreased, while we found no year-related differences in within-individual variance in adults (Table 4). Taken together, we interpret the decrease in within-individual variance found in juveniles as canalization of gizzard mass during ontogeny (Table 2 process 3).

The absence of age-related differences in repeatability of exploration behavior concealed underlying changes in variance components in both juveniles and adults. Within-individual variance in exploration decreased significantly between year-1 and year-2 for both juveniles and adults. Concomitant (non-significant) decreases in among-individual variance meant that there was no overall change in repeatability of exploration between year-1 and year-2 (Table 3). As we found no systematic change in average exploration behavior between year-1 and year-2 (Table 3, Figure 2), we rule out the possibility that the decrease in within-individual variance in exploration from year-1 to year-2 (Table 3) was the result of habituation to the experimental arena. Instead, the decrease in within-individual variance is consistent with the idea of canalization (Table 2, process 3). Because this decrease in within-individual variance was quantitatively similar for juveniles and adults, we consider the possibility that this decrease in within-individual variation may reflect a "time in captivity" effect, as opposed to a strictly developmental process (that would result in changes in the juvenile cohort alone). Since we found no increase in among-individual variance in exploration behavior in either juveniles and adults, we

suggest that among-individual differences in developmental plasticity in exploration were not at play during our experiments (Table 2, process 1).

Taken together, our results suggest that canalization may play an important role in the development of among individual differences for both gizzard mass and exploration. Importantly, our results also demonstrate that studying age-related differences in repeatability alone, without considering the differences in among- and within-individual variance components separately, is insufficient for studying developmental processes. This is because no overall change in repeatability can occur even when there are significant changes in the underlying variance components if these exhibit changes in the same direction.

We acknowledge that these trait-specific processes may be partially caused by aspects of our experimental design. Notably, our diet manipulation did not have any effect on mean exploration, even in juveniles (Figure 2). This indicates that, if there is a sensitive window during early development in which knots adjust their exploration behavior directly in response to changes in diet quality, it occurs earlier in development than the timescale of our experiments (i.e., 4 months of age). Consequently, we repeatedly induced within-individual shifts in gizzard mass, but not in exploration. This may have allowed for greater reduction in within-individual variance in exploration in the course of the study compared with gizzard mass. As with exploration behavior, there was no evidence that juveniles were more sensitive to diet manipulations than adults in terms of adjustment in gizzard mass (Table 4), because overall diet effects were similar for adults and juveniles within each study year.

By offering standardized aviary conditions, we deliberately separated the individual from its natural environment as a way of explicitly

controlling for factors that might influence physiology and behavior (Gibbons Jr. et al. 1994). Contrary to free-living knots, the birds in the context of our experiments lived in absence of competition for food and actual predation danger; these are potentially important factors known to contribute to among-individual variation (e.g., Bengston et al. 2014, Urszán et al. 2015, but see Horváth et al. 2017). The lower among-individual variance in gizzard mass and exploration in juveniles when compared to adults in year-1, indicates that among-individual variation increases over time in free-living knots. Our results therefore suggest that environmental conditions play a key role in shaping patterns of phenotypic (co-)variation in knots.

In addition, whereas co-variation between gizzard mass and exploration behavior has been reported in free-living knots (Bijleveld et al. 2014), we replicated the earlier finding that there is no co-variation between the gizzard mass and exploration in captivity (Bijleveld et al. 2014). At the same time, among-individual variation in both gizzard mass and exploration behaviour between year-1 and year-2 eroded in adults, and not in juveniles, suggesting that our captive conditions indeed removed a variable that generates and maintains among-individual variation (e.g. Archard and Braithwaite 2010, Fisher et al. 2015). However, our results are consistent with Bijleveld et al. (2014) and Oudman et al. (2016), who both found experimental manipulations of gizzard mass not to affect the expression of exploration behavior. Thus, repeatable variation in field gizzard mass may be considered 'pseudo-repeatable' (Niemelä and Dingemanse 2017; i.e., it is the outcome of repeatable exposure to different conditions such as habitat or diet). Indeed, the observed among-individual variance in gizzard mass was greater in two earlier studies where the choice for prey quality was greater (Bijleveld et al. 2014) and the time in captivity was shorter (Mathot et al. 2017).

At least two other studies have reported how within-individual canalization can contribute to increased repeatability. In sea anemones (*Actinia equina*), within-individual variation in the startle response decreased over time (Osborn and Briffa 2017). Similarly, a reduction of within-individual variance explained the strong age-related increase in repeatability in multiple behavioral traits in mosquitofish (*Gambusia holbrooki*) (Polverino et al. 2016, Table 1). We suggest that reduction in within-individual variation may be the outcome of Bayesian updating (Stamps and Krishnan 2017). With increased exposure to environmental cues, individuals may be more certain in their assessment of the environment. As their 'estimate' becomes more accurate, smaller phenotypic adjustments are needed (Stamps and Krishnan 2014b).

Returning to Waddington's (1942) metaphor of canalization, it is likely that the exact canalization process, and the adaptive value of within-individual canalization, varies between traits and that some traits are shaped more rigidly than others. The limited level of canalization of gizzard mass found here may be explained by the fact that there is strong selection to retain plasticity in gizzard mass. Red knots benefit by being able to fine-tune gizzard mass to seasonally changing diets and highly variable food conditions at the nonbreeding grounds (Zwarts and Blomert 1992, Piersma et al. 1993b, Battley and Piersma 2005a). The strong within-individual canalization of exploration on the other hand, might be the result of a few environmental switches during early development (Waddington 1942); it suggests that having a predictable expression of exploration behavior (which varies between individuals) is beneficial even early in life. The individual exploration behavior in red knots may well start as the hatchling chicks begin to forage and self-explore their tundra birthplaces.

Acknowledgments

Authorship statement:

EMAK and KJM conceived of and designed the experiment. EMAK, JBB, PM, DS, AD, and KJM collected the data. EMAK analyzed the data and wrote the manuscript with KJM and TP. All authors gave final approval for publication.

Electronic supplementary material

Table A1. Outcome of the multivariate model on the covariance between gizzard mass and (logit-) exploration behavior.

	Year-1		Year-2	
	among	**within**	**among**	**within**
Juvenile	-0.19 (-0.92,0.79)	0.07 (-0.12,0.26)	0.09 (-0.81,0.87)	-0.02 (-0.21,0.18)
Adult	0.19 (-0.77,0.92)	0.03 (-0.15,0.23)	0.16 (-0.75,0.87)	0.06 (-0.13,0.23)

Note. – Means (β) and 95% Credible Intervals (CI) of within-individual correlation estimates between gizzard mass (g) and (logit-)exploration behavior. To test whether individual differences in gizzard mass and exploration behavior could have been the result of state-behavior feedbacks between gizzard mass and exploration behavior during early ontogeny, we compared the within-individual correlation between gizzard mass and exploration between age groups and years. Within-individual correlation between gizzard mass and exploration in juveniles in year-1, but not in year-2, nor in adults or years, would have been indicative for state-behavior feedbacks during early ontogeny.

We constructed four separate bivariate models with gizzard mass and (logit) exploration behavior as response variables. These models were constructed with a Gaussian error distributions. As we were only interestd in the covariance structure, no fixed effects were included. A random effect bird ID was included to be able to separate within- and among-individual variance components of gizzard mass and exploration. Models were run for 1003000 simulations with a burnin of 3000 and an thining interval of 1000, yielding a total of 1000 estimates from which to contruct posterior distributions.
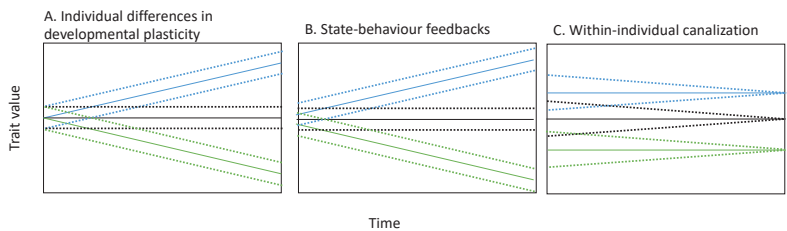
Figure A1. Visual representation of the three processes underlying age related increase in repeatability. Visual representation of three mechanisms underlying age-related increase in repeatability. Each color represents a different individual. Solid lines indicate mean trait values per individual, and dotted lines represent within-individual variance in trait values (i.e., residual variance). Panels A and B differ only in that state-behavior feedbacks require some initial among-individual differences, though these can be small, and may arise due to a chance events.
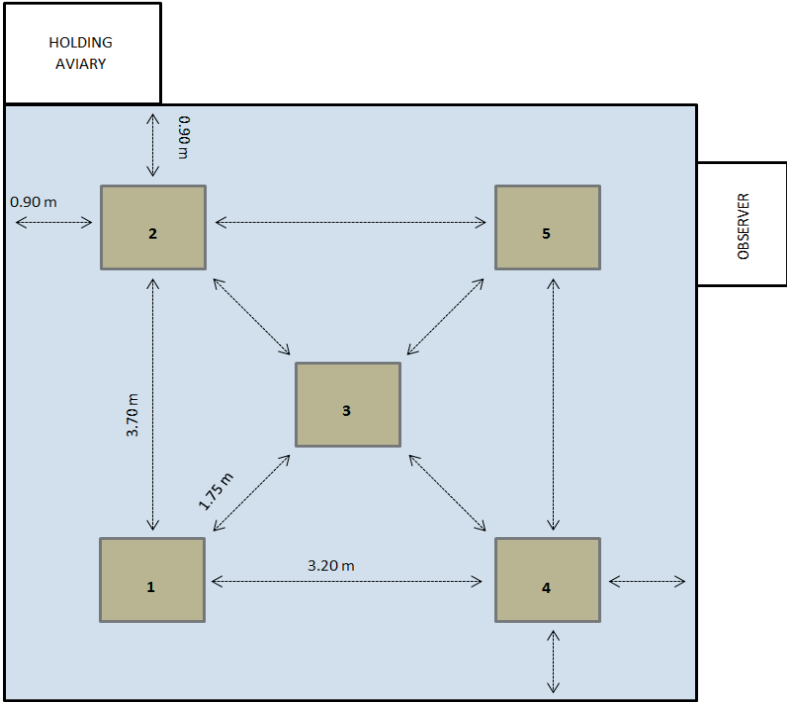
Figure A2. Layout of the experimental arena. Experimental birds were released individually from the holding aviary. Behavior was observed and digitally scored by one observer from behind a one-way-mirror next to island 5.

BOX 2

INDIVIDUAL DEVELOPMENT OF MIGRATORY ROUTINES IN RED KNOTS *(CALIDRIS CANUTUS CANUTUS)*

Eva M.A. Kok

Box 2

Out of more than 12000 red knots which have been colour-ringed by the NIOZ-team over the past 25 years, primarily at Banc d'Arguin in Mauritania and in the Wadden Sea, red knots (*Calidris canutus*) of known subspecies are only very rarely seen at locations in the 'wrong' part of the flyway. There are for example two records of birds that are ringed in winter in Western Europe (when only red knots of the *C. c. islandica* subspecies are supposed to be present) were resighted in Poland during autumn migration (where only *C. c. canutus* should occur). Similarly, a handful of knots ringed in Mauritania (*C. c. canutus* subspecies) have been seen in Iceland and northern Norway (where only *C. c. islandica* should occur; Davidson and Wilson 1992, Piersma 2007, Buehler and Piersma 2008). Only once a chick that has been ringed on the breeding grounds in Greenland in 2003 has been observed in Mauritania 2006 (J. ten Horn, T. Piersma and J. Wilson, pers. comm.). It is this kind of observations that make us wonder how consistent knots are in their migratory routines, and how juvenile red knots know to find the appropriate route when they migrate for the first time.

Studies on other bird species showed that individual migratory strategies form during development (Lok et al. 2011, Gill et al. 2014, Verhoeven et al. 2018, Oudman et al. 2020). After fledging juvenile knots migrate south in the absence of adult knots who have left the breeding grounds before them (Meissner et al. 2009). The fact that individuals, after they migrate south in their first year, do not return to the breeding grounds to breed until their third summer, after which they are thought to breed every year, is indicative for a developmental process towards a migratory strategy (Piersma and Davidson 1992).

Here we will use the existing differences between the global distributions of *C. c. islandica* and *C. c. canutus* (see Figure 3 in Chapter 1) to investigate if we can find experimental evidence that contextual differences during ontogeny affect the 'shape' of the eventual individual migratory routine.

In 2015 and 2016 we captured juvenile knots, at what is presumed to be their first stopover on intertidal mudflats, near Gdansk Bay in Poland during their first southward migration (Meissner 2005, Meissner et al. 2009, van Gils et al. 2016). Red knots that stop in Gdansk bay during southward migration predominantly belong to the *C. c. canutus* subspecies (Meissner et al. 2009 but see Meissner and Gromadzka 2006).

These knots were consequently deprived of their natural, subspecies-specific, environmental stimuli for the next three or four years as they were kept at the NIOZ Experimental Shorebird Facility on Texel. Held captive within the natural wintering range of *C. c. islandica* knots and in mixed flocks with individuals of the *C. c. islandica* subspecies, one may argue that we 'imprinted' the *C. c. canutus* juveniles as young *C. c. islandica* (if there is such a thing as migrational imprinting, see Piersma 2011a).

During southward migration, the peak transition date through the Wadden Sea for knots belonging to the *C. c. canutus* subspecies is between 8 and 18 August (Piersma and Davidson 1992, Piersma et al. 1993a, Nebel et al. 2000). To investigate if the manipulated *C. c. canutus* knots were able to resume 'normal' migratory behaviour, we released these *C. c. canutus* after 3 or 4 years in captivity in the Wadden Sea just before the appropriate migratory period (on 2 August 2019).

Besides color rings for remote individual identification 30 of the 48 knots were released with a glued-on WATLAS transmitter (weight = 5 g, lxwxh = 10x10x3 mm). The WATLAS tracking system was developed to automatically locate, identify and store locations of dozens of individuals carrying a radio transmitter within a predefined geographic region confined by a network of receiver stations in the Dutch Wadden Sea. The detection range of a WATLAS transmitter is limited by 'the line of sight' between the transmitter and the

Box 2

receiver station. This range consequently varies a lot depending on the position of the receiver tower (or handheld) and the altitude of the transmitter, i.e. if the bird is on the mudflats or in flight, but was estimated to be able to reach a distances up to 20 kilometer. Within the WATLAS perimeters the location of the transmitter is calculated by means of the time difference of the arrival of the transmitted signal at the different receiver stations at known locations (MacCurdy et al. 2019). In 2017 the WATLAS system was made up of 5 receiver stations around the island of Griend. The system was operational until roughly 1 month after release of the red knots reported in Table 1 and 2. In 2019 26 receiver stations formed the WATLAS system, this time almost covering the whole western Wadden Sea. In 2019 the receiver network was maintained at full strength at least until September 2019, after which is was kept operational all winter in a reduced setting.

To separate the ontogenetic effect of interest from a possible captivity-effect, we compared the reported color ring resightings within five months after release and correlating WATLAS recordings to five other groups of knots (Table 1 and Table 2). We also intended to keep and follow a group of adult *C. c. canutus* knots for comparison but we were not able to keep sufficient numbers of birds. Unfortunately adult *C. c. canutus* proofed difficult to capture and turned out to be inapt for long term captivity. Adult *C. c. canutus* are difficult to capture during southward migration because the majority of knots fly south from the breeding grounds to Western Africa with fewer intermediate stopovers (Nebel et al. 2000). Contrary to the regular occurrence of juveniles in Gdansk bay the irregular and short term occurrence of adult *C. c. canutus* together with the poor local feeding conditions suggest that the Baltic coast is an emergency feeding place for adult *C. c. canutus* (Meissner 2007, Meissner et al. 2009, van Gils et al. 2016), instead adult *C. c. canutus* are known to make their first stop in the Wadden Sea (Nebel et al. 2000). The observation, that the *C. c. canutus* adults that we managed to capture in the eastern part of the Dutch

Wadden Sea (n = 8, identified based on high body mass and absence of wing molt, see Nebel et al. (2000); and in one occasion because it was recaptured after originally being ringed in Mauritania) and Poland (n = 11) appeared much more prone to disease in captivity than adult *C. c. islandica* and juvenile *C. c. canutus* was unfortunate, although it hints at potentially interesting immunological facts about red knots at emergency staging sites.

Results

Banc d'Arguin

During a field expedition between 20 November and 7 December 2019, approximately four months after the average arrival date of the subspecies in this area, we tried to locate the manipulated *C. c. canutus* knots at the main wintering site Parc National du Banc d'Arguin (PNBA, Mauritania; Piersma and Davidson 1992). In the past 21 years 816 knots ringed in the Wadden Sea have been resighted in PNBA (J. ten Horn pers. comm.). Daily ring reading sessions by 11 experienced ring readers during the 2019 field expedition allowed for the individual identification of 500 color-ringed knots, however none of the manipulated *C. c. canutus* knots were resighted.

We also tried to locate the experimental knots equipped with WATLAS transmitters (n = 30) by means of a handheld receiver. This receiver allows the operator to detect any WATLAS transmitters within 'the line of sight' of the antenna. The detection range of a WATLAS transmitter varies a lot depending on the position of the receiver tower (or handheld) and the altitude of the transmitter, i.e. if the bird is on the mudflats or in flight, but was estimated to be able to reach a distances up to 20 kilometer (F. van Maarseveen pers. comm.). Between 23 November and 7 December 2019 researchers visited most of the important high tide roosts in the area twice (Nair, Arel, Tidra, and Gibene). The Baie d'Aouitif and high tide roost Ebel Kheaiznaya were

Box 2

covered almost continuously in this period by means of an antenna attached to the roof of the field station near the village of Iwik and car surveys down the bay. Despite this great effort, none of the tagged knots were detected.

Dutch Wadden Sea

Obviously, absence of confirmation of their presence in PNBA is not the same as confirmation of their absence. Therefore we also investigated if the manipulated *C. c. canutus* knots left the Wadden Sea at the time that wild *C. c. canutus* pass through on their way to Mauritania we compared the number of color ring resightings as well as WATLAS detections between the first two weeks after release (when we predict the *C. c. Canutus* knots to be detectable) and for the period after that (when we expect the *C. c. canutus* knots to have left the Wadden Sea; Table 1; Group A).

The observation that at least half of the tagged *canutus* knots were resighted in the Dutch Wadden Sea up to five months after the peak southward migration through this area (n= 27; see Table 1 and 2; Group A) suggests that the *C. c. canutus* knots kept in captivity on Texel did not readily migrate to their normal West-African wintering grounds. Also the WATLAS system still reports the presence of *C. c. canutus* two weeks after release. In fact, the detections of a group of wild adults (*C. c. islandica*) that was released between 4-8 August 2019 on Richel show a similar pattern (Table 1; Group B).

A preliminary analysis of the local movements of the *C. c. canutus* knots within the Wadden Sea in the first 5 months after their release indicates that their habitat selection criteria were wanting. Previously color marked knots caught in the Wadden Sea that received no additional treatment were most frequently reported from the main high tide roosts in the Wadden Sea (Richel and Griend) after release. The same applies to wild knots that were marked in the Wadden Sea

in August 2019 (n = 43, Table 2; Group B) and in 2016 and 2017 (n= 824, Table 2; Group C) and resighted for the period of study (between August 2019 and January 2020). The *C. c. canutus* knots from long-term captivity were repeatedly reported at locations where wild knots were rarely seen (Table 2), among which at least five locations that seem unsuitable for red knots in terms of habitat characteristics (J. ten Horn, A. Dekinga, J.A. van Gils and R.A. Bom pers. comm.).

To investigate if these unexpected movement patterns could be the result of an unwanted 'captivity' effect, rather than 'imprinting' we also included a comparison between groups of juvenile and adults knots of the *C. c. islandica* subspecies that had been held captive under similar circumstances for two years before they were released in 2017 on Griend (Tables 1 and 2; Groups D and E). The juveniles and adults of the *C. c. islandica* subspecies showed no considerable variation in spatial distribution within 5 months after release in the Dutch Wadden Sea. Also, none of these knots have been seen at the odd places that the *C. c. canutus* were reported from.

Conclusion

Our admittedly incomplete observations are consistent with the idea that withholding the first southward migration to West Africa and captive residency during subsequent years led to an 'imprinting' of *C. c. canutus* knots on the wintering grounds of *C. c. islandica*. It is also possible that the *C. c. canutus* knots did not reach West Africa (yet) because they are generally confused by their previous long-term confinement and subsequent very recent release. Future resightings in West Africa and the Wadden Sea will allow us to calculate the general survival of these released *canutus* knots and/or they may reveal possible delayed arrival in West Africa.

Box 2

However, the pattern of habitats in which the released *C. c. canutus* knots were resighted locally suggest that not learning the habitats for three years may have prevented them from easily learning them from free-living conspecifics of either subspecies immediately after release. Thus, exposure to natural environmental cues during ontogeny may be crucial for the development of 'normal' routines.

Acknowledgments

Table 1. Local resighting and WATLAS detection rate of red knots in the Dutch Wadden Sea within the first 5 months after release.

	2019	2019	2017	2017	2017
Group	A[1]	B[2]	D[3]	E[3]	F[4]
Subspecies	C. c. canutus	C. c. islandica	C. c. islandica	C. c. islandica	C. c. islandica
Age at capture	juvenile	adult	juvenile	adult	adult
Captive period	3 or 4 years	2–5 days	2 years	2 years	-
Sample size	nTotal = 46 nTransmitters = 30	43	24	19	30
Resightings (NL)					
Within 2 weeks	0.28	0.07	0.29	0.58	0.87
2 weeks – 5 months	0.46	0.42	0.42	0.63	0.87
Proportion of knots detected					
WATLAS detections (NL)					
Within 2 weeks	0.77	0.88	0.92	1	1
After 2 weeks	0.53	0.73	0.33	0.47	0.70

1= This group of juveniles (*C. c. canutus*) was caught in Gdansk bay (Poland) in 2015 and 2016 and released 3 or 4 years later; on 2 August 2019 in the Wadden Sea (Richel). Only part of this group was equipped with a WATLAS Transmitter (n = 30). 2= This group of wild adults (*C. c. islandica*) was capture on 3 August 2019 in the Wadden Sea (Richel). 3= These groups of juveniles and adults (*C. c. islandica*) were caught in the Dutch Wadden Sea in 2015 and released on 15 September 2017 in the Wadden Sea (Griend). 4= This group of adults (*C. c. islandica*) was captured and released on 23 August 2017 in the Wadden Sea (Richel). Sample size = number of unique birds per group, Resightings (NL) = the proportion of the cohort that was resighted in the Dutch Wadden Sea for the given time period, WATLAS detections (NL) = the proportion of the cohort that was detected within the perimeter of the WATLAS system for the given time period.

Box 2

Table 2. Local resighting rate of red knots in the Dutch Wadden Sea. Proportion of knots resighted per location within the first 5 months after release.

Group	A[1]	B[2]	C[3]	D[4]	E[4]	F[5]
		2019			2017	
Subspecies	C. c. canutus	C. c. islandica	NA (mainly C. c. islandica)	C. c. islandica	C. c. islandica	C. c. islandica
Age at capture	juvenile	adult	NA (mainly adult)	juvenile	adult	adult
Captive period	3 or 4 years	2-5 days	0 days	2 years	2 years	-
Sample size	46	43	824	24	19	30
Resighted (NL)	nBird = 27, nRes = 106, nBird/Loc = 61	nBird = 19, nRes = 51, nBird/Loc = 25	nBird = 153, nRes = 295, nBird/Loc = 190	nBird = 10, nRes = 41, nBird/Loc = 12	nBird = 12, nRes = 46, nBird/Loc = 15	nBird = 30, nRes = 216, nBird/Loc = 35
Location			Proportion of cohort resighted per Location			
Richel (release site 2019)	0.15	0.28	0.29	-	-	0.11
Griend (release site 2017)	0.18	0.64	0.61	0.67	0.73	0.86
Terschelling Oosterend	0.05	-	0.05	-	-	-
Ameland Hollum	-	-	0.03	-	-	-
Terschelling Striep	0.13	-	0.01	-	0.07	-

Table 2. Continued

Group	2019			2017		
	A[1]	B[2]	C[3]	D[4]	E[4]	F[5]
Harlingen	0.13	–	–	-	-	–
Terschelling 't Sehaal	0.10	–	–	–	–	–
Terschelling De Keag	0.05	-	0.01	–	–	–
Balgzand Normerven ewww.o.	0.03	0.04	-	–	–	–
Texel Schorren	0.03	–	0.01	0.17	0.07	-
Vlieland Strand Paal 21	0.03	–	–	–	–	–
Ameland strand van Nes	0.03	–	–	–	–	–
Friese kust Zurich	0.03	–	–	–	–	–
Kornwerderzand	0.03	–	–	–	–	–
Posthuiswad Vlieland	0.03	–	–	–	–	–
Texel de Petten	0.03	–	–	–	–	–
Texel de Cocksdorp	–	0.04	-	0.08	0.07	0.03

Box 2

Table 2. Continued

Group	2019			2017		
	A[1]	B[2]	C[3]	D[4]	E[4]	F[5]
Vlieland						
Noordzeestrand thv						
Posthuys	0.03	–	–	–	–	–
Oosterschelde						
W.polder	–	–	–	–	0.07	–
Oosterbierum	–	–	–	0.08	–	–

1= This group of juveniles (*C. c. canutus*) was caught in Gdansk bay (Poland) in 2015 and 2016 and released 3 or 4 years later, on 2 August 2019 in the Wadden Sea (Richel). 2= This group of wild adults (*C. c. islandica*) was capture on 3 August and released between 4-8 August 2019 in the Wadden Sea (Richel). 3= This group of wild knots predominantly consisted of adults (>90%) and was captured and marked in the Dutch Wadden Sea in 2016 and 2017 and resighted in the Dutch Wadden Sea between August 2019 and February 2020. 4= These groups of juveniles and adult red knots (*C. c. islandica*) were caught in the Dutch Wadden Sea in 2015 and released on 15 September 2017 in the Wadden Sea (Griend). 5= This group of adult red knots (*C. c. islandica*) was captured and released on 23 August 2017 in the Wadden Sea (Richel). Sample size = number of unique birds per group, Resightings = resightings in the Dutch Wadden Sea, nBird = the number of unique birds resighted per group, nRes = the total number of resightings per group, nBird/Loc= the unique number of resightings per site per bird per group. The proportions indicate the proportion of birds resighted per location (nBird/Loc), locations in italics (n=5) are considered less suitable for red knots because they are situated at odd places or due to the presence of sandy beaches, rather than mudflats (A. Dekinga, J. ten Horn, J. A. van Gils and R. A. Bom pers. Comm.).

EXPERIMENTAL TESTS OF A SEASONALLY CHANGING VISUAL PREFERENCE FOR HABITAT IN A LONG-DISTANCE MIGRATORY SHOREBIRD

Eva M.A. Kok, Jerry A. Hogan and Theunis Piersma

Migratory shorebirds show highly organized seasonal cycles in physiological and morphological traits (body mass and composition, plumage, hormone levels, etc.), which in captivity is accompanied by restless behaviour at times when free-living birds would start migration. We introduce the idea that a seasonally changing preference for habitat could motivate migrants to embark on migration and that this cognitive process could also guide them to seasonally appropriate places. We explored this by testing whether red knots (*Calidris canutus*), which also in captivity maintain marked circannual phenotypic rhythms, show evidence of seasonal change in preference for pictures of seasonally appropriate habitats. We first developed a method to verify if red knots are able to memorize and discriminate contrasting pictures projected by LCD projectors. This was followed by two different experiments in which we tested for a seasonally changing preference for breeding or non-breeding habitat. When carried out during the pre-breeding season, the red knots are expected to prefer pictures of mudflats, their non-breeding habitat. At the start of the breeding season, they should prefer pictures of the tundra breeding habitat. We established that knots are able to distinguish and memorize projected images. We failed to demonstrate the predicted change in vision-based habitat preference, but for reasons of test design we do not interpret this as a strong rejection of the hypothesis. Instead, we suggest that experiments with greater numbers of individuals tested once, perhaps in combination with the provision of additional cues like smells and sounds, will help the development of these ideas further.

Introduction

The ecological opportunities provided by the seasonally changing environmental conditions on Earth have favoured an enormous variety of seasonal migration phenomena (Alerstam 1990b, Newton 2008). The animals engaging in such long-distance migrations perform amazing feats of endurance exercise (Piersma 2011b), and navigation (Muheim 2006, Åkesson and Hedenström 2007, Ritz et al. 2010, Mouritsen 2018, Muheim et al. 2018). What seasonal migrants have in common is the circannual steering of relevant physiological processes in relation to navigation (Pinzon-Rodriguez et al. 2018) and the circannual expression of labile physiological and morphological ('physiomorphic') traits that facilitate endurance exercise during migration as well as behaviour for survival and reproduction during the appropriate seasons (Gwinner 1996, Bijleveld et al. 2015, Karagicheva et al. 2018). Physiological preparation for migration in spring is associated with high corticosterone levels (Piersma et al. 2000, Landys-Ciannelli et al. 2002, Eikenaar et al. 2014), increases in restless behaviour (Gwinner 1986, Zúñiga et al. 2016), and enhanced cognitive performance (Rattenborg et al. 2004). This prompted the question if migrants, along with the well-studied suite of corporal and cognitive changes, would show corresponding seasonal changes in habitat preferences?

Memory may be an important motivator of goal-oriented movements (Bennett and Tang 2006, Fagan et al. 2013, Bracis and Mueller 2017, Rodríguez et al. 2017). Garden warblers (*Sylvia borin*), who were trained to associate temporal and spatial changes in food availability in an experiment where food was available temporarily but predictably in multiple connected rooms, learned to visit a specific foraging site at a given time of the day (Biebach et al. 1989). Such capacities would be extremely useful for shorebirds such as the subjects of this study, birds depending on a circatidal cycles for the accessibility of its food

resources (Bulla et al. 2017). Similarly, associating circannual rhythms with spatial changes in habitat characteristics during migration may be adaptive.

Memory-motivated movements are most likely to occur in long-lived species for which the scale of the orientation by individual animals is greater than their perceptual range, for example during long-distance migration (Mueller and Fagan 2008, Berbert and Fagan 2012). In a recent review, Winger et al. (2018) suggested that seasonal migratory routines have evolved as a result of the fitness benefits associated with returning to familiar summer- and winter areas rather than previously unvisited, 'new', areas. The ability of an individual to return to a previously visited site implies the use of a cognitive 'concept of place'. The occurrence of an long-lasting spatial memory may be especially adaptive in long-distance migratory birds: they move between sequences of distant habitats for which no up-to-date information is available at the time of departure towards any of them (Cristol et al. 2003, Mettke-Hofmann and Gwinner 2003, Pravosudov et al. 2006, Berbert and Fagan 2012, Winkler et al. 2014).

Memory could also support motivation. The possibility of mental images of preferred habitats which change with the seasons brings up the notion of 'home sickness', a longing for a familiar but distant place. Among other causes for spring migration such as; the availability of food and extended day length at northern latitudes, Linnaeus (1757) listed feelings for home. In 1928, Allard published a review on possible directive stimuli that urge and guide migratory birds, between breeding and non-breeding grounds. Possible motivational forces for northward migration include "a longing for light" (previously defined as a driving force by the Finish ornithologist Johan Ludvig Runeberg) and a 'drawing force' to go home (contrived by English,1923, cited in Allard 1928). Here we elaborate on the idea that migratory movements may be motivated (*sensu* Hogan 2017) by seasonally changing habitat preferences. The

tests for seasonally changing habitat preferences presented here were inspired by a study on dark-eyed juncos (*Junco hyemalis;* Roberts and Weigl 1984). By their seasonally changing selection for pictures of either breeding or non-breeding habitat, Roberts and Weigl suggested that juncos possess a vision-based concept of habitat. We developed an experimental protocol to test if red knots (*Calidris canutus*) are able to memorize and distinguish projected pictures of contrasting landscapes. Once this was established, we explored whether red knots showed changing preferences for pictures of seasonally appropriate habitats.

General Methods

Red knots, hereafter knots, are sandpipers that migrate many thousands of kilometres between distinct non-breeding (coastal mudflats) and breeding habitats (high arctic tundra) and show strict circannual rhythms in plumage, body mass, and other physiomorphic traits, even when held in captivity (e.g. Piersma 2007, Buehler and Piersma 2008, Karagicheva et al. 2016).

Animal husbandry

The knots studied here were captured with mist nets in September 2012 and between August 2015 and February 2016 at different high-tide roosts in the Dutch Wadden Sea. After capture, the knots were aged on the basis of plumage characteristics either as juvenile, second calendar year, or older (i.e. adult). The knots were colour-ringed for individual identification. All knots belonged to the *islandica* subspecies (Piersma 2007), this subspecies breeds on tundra in northeast Canada and northern Greenland and winters in northwest Europe (Davidson and Wilson 1992).

On the island of Texel (53°00'N, 04°47'E), in the Experimental Shorebird Facility of the NIOZ Royal Netherlands Institute for Sea Research,

the birds were kept in flocks of approximately eight individuals. The facility is located within the natural geographic wintering range of the subspecies. The outdoor aviaries were 4 m long, 1.9 m wide, and had a sloping roof at 2.3 m height at one side and 1.9 m at the other. All aviaries contained smoothly-coated concrete floors constantly irrigated with running seawater and, at the back of the cage, a mudflat basin (1.9 m x 1.0 m) filled with Wadden Sea sand and running seawater. The knots experienced local ambient temperatures and a seasonally changing photoperiod through a screened window (1.9 m x 0.9 m) in the back wall. When not in the experiments, knots had *ad libitum* access to Trout food pellets (Produits Trouw, Vervins, France) and fresh water for drinking and bathing in a separate tray.

Every week, while the aviaries were cleaned and disinfected with chlorine, the general health of the knots was checked and their body mass and body moult scored (Milot et al. 2014). We used plumage score to indicate the extent of physiological preparation for breeding (Karagicheva et al. 2016). Body plumage was scored on a scale from 1-7. In winter knots show white breast plumage and a grey back (indicated with 1). In the weeks before the breeding season they moult into a rufous red breast plumage with much darker back feathers (indicated with 7 when in full breeding plumage). This transition takes a few weeks and the final extent of breeding plumages also depends on the age and the body condition of the bird. When the experiments were finished, the knots were released near the capture site in the Wadden Sea.

Experimental room

All experiments were performed in an indoor experimental room measuring 7 m by 7 m, with a height of 2.80 m. The 'floor' consisted of two areas of 'mudflat' separated by a 'gully' (see Figure 1). The two mudflats could be exposed or submerged as the water table could be raised to a depth of 30 cm. The room had no outside facing windows

and was dimly lit during experiments to ensure projection quality. Projections were made on either side of the experimental room on two screens of water-resistant multiplex plywood coated with a thin layer of white epoxy.

Experimental rationale

We report on four experiments performed from 2012-2015. We first developed an experimental protocol to establish if knots are able to distinguish projections of natural landscapes (Discrimination Test I). Once this was established, we continued with two experiments to show seasonally appropriate preferences for habitat (Preference Tests I and II). In view of our failure to demonstrate seasonally changing habitat preferences, and indeed the absence of a clear habitat preference in the two tests in general, we again verified that knots can show us that they are able to distinguish between projected images in the experimental set-up and with the procedures chosen; this was Discrimination Test II.

Experimental procedure

Although these experiments served different goals, they were all based on the dichotomous preference test (van der Meer 1992); the 'competing' pictures were semi-randomly distributed between two opposing screens (according to a balanced design but in unpredictable order for each bird).

Every test included the release of a single knot from a blinded wooden cage (1 m by 1 m by 1 m) with a smaller (60 x 40 x 33 cm l x b x h) inset, placed in the middle of the wall at equal distance from the two projection screens (Figures 1-4). The front panel of this cage consisted of a see-through screen. Before the start of a trial, the focal bird was caught from its home aviary and its body mass was taken before it was placed into the release cage. The front screen of the release cage was opened remotely with a pulley system to allow the bird to leave and indicate the image of choice (see below for the exact definition of the

images of choice for each test). When a trial ended, the knot was gently herded back to one of the side aviaries and returned to its home aviary (see Figures 1-4).

Analyses

For all four experiments we ran univariate models using the *glmer()* function from the lme4 package (Bates et al. 2015) with a logit link (family = 'logit') to model the binomial response variable. To investigate the possibility that individuals differ in their performance, 'individual identity' was included as a random intercept in all models. When we found a value of zero for among individual variance, we confirmed the negligible effect size of bird ID by fitting it as a fixed effect (results not shown). To draw inferences based on simulated posterior distributions, Bayesian statistics were used.

To simulate values of the posterior distribution of the log-odds of the correct choice given the model parameters (Gelman and Hill 2007), we used the *sim()* function of the arm package (Gelman and Su 2016). We used 95% credible intervals (CIs) around the mean (β) fixed effects that were extracted based on 1000 simulations using the MCMCglmm package (Hadfield 2010). The 95% CI indicates a margin of error in terms of a range of plausible values for β, indicating that we are 95% confident that our CI includes the actual effect size (Cumming and Finch 2005). In other words, a 95% CI that does not overlap zero would be considered significantly different from zero ($p < 0.006$) in a null hypothesis testing framework (Cumming 2009). For each test we tested for autocorrelation between subsequent replicates by means of the function *runs.test()* from the tseries package (Trapletti and Hornik 2018). All analyses were done in the R (v3.6.3) statistical environment (R Core Team 2020).

Discrimination Test I

Methods

Several bird species have been shown to memorize and distinguish projected images (Pietrewicz and Kamil 1977, Brown and Dooling 1993, Bovet and Vauclair 2000, Watanabe 2001). However, because no picture-habitat recognition experiments had been done on sandpipers nor on any other shorebirds, we first developed a testing protocol to determine if knots are able to distinguish between projected images.

During this experiment the knots were housed in the experimental room and only temporarily removed during testing. In this test the experimental room was set up such that birds could walk (and optionally fly) to their preferred picture. Pictures were projected on opposite sides of the aviary using two LCD projectors (projector properties- type: BenQ MP615, SVGA: 800x600, light intensity: 2000 lumen, contrast: 1000:1, mass: 2.7 kg). The projected areas measured 2.5 m high and 5 m wide (Figure 1).

Two contrasting pictures (1024x768 pixels) of possible non-breeding habitat were selected. One was of an open shore in the Dutch Wadden Sea (mudflat), the other showed the tropical forest margin of a mudflat in the Archipelago dos Bijagos, Guinea- Bissau (forest) (Figure 1). Feeders were placed in front of the two pictures, but only one of them contained the food reward (Trouvit food pellets). Two knots were trained to find food pellets in a feeder in front of the picture of forest and the other two birds were trained to find food near the mudflat picture. To avoid the possibility that knots made a choice based on specific colours rather than the scene, the pictures were equalized with respect to reflected colour load using the function match colour in Adobe Photoshop (Cs6 Extended).

Tests were preceded by two days of training during which either the picture of the mudflat or the forest was projected on both sides with

ample pellets in both feeders in front of the projections. During the morning, birds were introduced to the room in pairs. Later, they were individually trained for the release procedure, the reward locations and trial closings. At the start of each test, the focal knot was left in the release box for one minute to observe both projections. When the front panel was opened the bird usually left the box immediately. Once the bird approached one of the two feeders up to less than 5 cm, the choice was made. When this was the 'correct' feeder (i.e. the feeder containing food), the bird was allowed to take a few pellets from it. The trial ended when the bird lost interest in the feeder (usually after less than one minute).

Birds were tested for 21 days between 9 March and 12 April 2013 with a total of 86 trials for all birds. All birds were tested before the next trial was begun. During the days of experiments, the birds received no other food than what they could eat at the feeder next to the rewarded picture. However, whenever a bird would drop below a critical threshold body mass of 100 g, additional trout pellets were offered between series of experiments. Especially during the first days of testing the birds were not always motivated to make a choice, probably because they were not hungry enough. These trials were excluded from analyses (See Appendix S1 Table S1).

To test whether birds successfully learned and distinguished projections (i.e. if the proportion of correct choices differed from random choice), we initially ran univariate models without fixed effects and a binominal response variable (0 = false, 1= correct; indicating if the bird had chosen the image it was trained for). To account for the possibility that the birds might have an initial preference for one side of the room, regardless of the picture presented, we subsequently added a fixed effect for the 'side' at which the 'correct' image was projected (0 = south side of the room, 1 = north side of the room). Additionally, the fixed effect 'group' (forest = 0, mudflat = 1) was included to investigate if birds assigned to different groups performed differently.
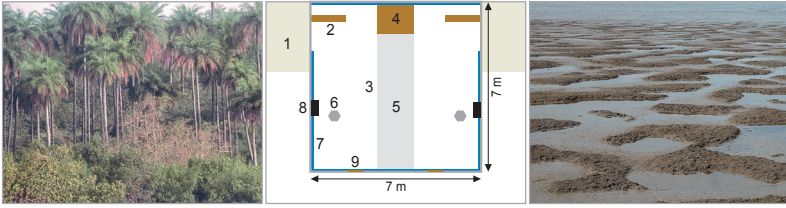
Figure 1. Experimental set-up Discrimination Test I. From left to right; picture of a coastal forest in Guinea-Bissau; The experimental shorebird facility: (1) side-aviary, (2) access steps aviary, (3) mudflat, (4) release cage, (5) concrete lane, (6) feeder, (7) projection screen, (8) projector, (9) entrance door; picture of Dutch Wadden Sea. During the experiments the side on which either of the projections were made was varied randomly.

Results

Three of the four birds chose the correct side consistently above chance levels. Overall, the knots learned to discriminate between images (after back transformation from the intercept model: β= 65%, 95% CI = 56%, 71%, Figure 5). Birds assigned to the forest group performed significantly better than the mudflat group (Table 1, Appendix S1 Table S1). This mainly resulted from the fact that one individual trained on the picture of a mudflat never learned to discriminate (Table 1, Appendix S1 Table S1). Choices were biased towards a preference for the south side of the room (Table 1). Overall, the performances did not improve with successive trials (one-sided Runs Test: Z = -1.05, p = 0.15, Appendix S1 Table S1).

Preference Test I

After establishing that knots are indeed able to distinguish between what must be memorized projections, we continued to test whether knots change their vision-based habitat preference depending on time of the year and physiological state. During testing, the knots were offered the choice between simultaneously projected pictures of a non-breeding habitat (intertidal mudflat) and a breeding habitat (tundra). Tests were carried out in early May (when knots were still preparing for migratory departures) and again in early June (when they are fully ready to take off to the breeding grounds). We predicted that the knots would show a preference for the mudflat picture during the pre-breeding season and for the tundra picture in the breeding season.

Methods

Before testing, the 13 birds studied in this experiment were housed in outside aviaries where they were exposed to seasonally appropriate ambient temperature and photoperiod to ensure natural phenotypic circannual rhythms. In March 2014 they were moved to similarly sized indoor aviaries, where the air temperature was held constant at 12°C and photoperiod followed the outdoor situation. In mid-April the knots were moved to the indoor experimental room. Here the photoperiod followed the outdoor situation by means of a 'day-light' lamp.

The design of the experimental room was very similar to the design in Discrimination Test I. However, water level in the room was raised so that birds had to fly the 3 m from the release box to a 1 m² sandy patch in front of the projections (Figure 2). During the tests a single picture of either mudflat or tundra was used repeatedly. Both pictures were taken at a similar angle and had the top one-third taken up by blue sky. During five days of training in late April, the knots were first introduced to the room as a group and later individually.

Testing took place from 1-10 May 2013 and again from 3-12 June 2013. Each bird (n = 13) was given one trial per day for ten consecutive days (n = 10) in both testing periods. The rest of the flock was confined to one of the two side aviaries during testing (Figure 2). During tests the focal bird was left in the release box for one minute before the screened panel was opened. The bird indicated its preference by flying towards its image of choice and landing on the patch in front of it. After five minutes the trial ended; if the bird had not left the release box by then, the trial was excluded from analyses (see Appendix 1 Table A2). During testing no food reward was offered, the only reward being the chance to stand on the sandy patches near one of the two projections.

To test whether knots showed seasonally changing preference for habitat we constructed univariate models with a binomial response variable for habitat (0 = mudflat, 1 = tundra). Besides the inclusion of a fixed effect for the projection side and a random effect bird ID (see the method section for Discrimination Test I for more details) we modelled habitat preference as a response to season (0 = breeding-, 1 = pre-breeding season). Weekly obtained plumage scores were interpolated for intermediate experimental days to indicate the extent of physiological preparation for the breeding season. Because not all birds reached full breeding plumage (score = 7, see Appendix S1 Figure S1 and S2), we included a bivariate fixed effect for the presence or absence of breeding plumage (1 = for plumage scores ranging from 1 to 4, and 0 = plumage scores between 5 and 7) to examine the effect of variation in breeding plumage scores on habitat preference.
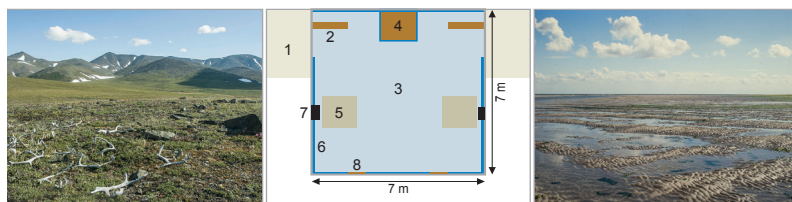
Figure 2. Experimental set-up for Preference Test I. From left to right: picture of breeding ground (known knot habitat in the arctic tundra in Chukotka, Anadyr); The experimental shorebird facility: (1) aviary, (2) access steps aviary, (3) sea water, (4) release cage, (5) sandy patch, (6) projection screen, (7) projector, (8) entrance doors; picture of wintering ground (Dutch Wadden Sea). During the experiments the projection side was designed to vary randomly.

Results

The knots showed a mean preference of 55% for mudflat during both the pre-breeding and breeding season (Appendix S1 Table S2), so no seasonal change in habitat preference was found (Figure 6, Table 2). Individuals did not differ in their seasonal preference for habitat (Table 2; 95% CI for bird ID is centred on 0). Especially during the breeding season, in some trials birds refrained from making any choice at all (missing values during the pre-breeding season n= 11, breeding season n= 25, see Appendix S1 Table S2); this lowered sample sizes considerably. There was also no association between the extent of breeding plumage and habitat preference, and no bias was found towards one side of the experimental room (95% CIs are centred on 0, Table 2). Also, we found no autocorrelation between successive trials (two-sided Runs Test: Z = 1.52, p = 0.13).

We also considered the possibility that the percentage of time spent near either of the two images may hold additional information on preferences. Therefore, we reran the analyses with a novel binary response variable indicating if a bird spent more than 50% of the time just in front of the Tundra image (1), or more than 50% of the time immediately in front of the Mudflat image (0). These analyses, based on the percentage of time spent near either image (rather than the first choice for one of them), yielded similar results (not shown); note that

only in a few trials birds switched between patches (n = 34/224).

Preference Test II

After having established that knots were able to memorize and distinguish between projected landscape pictures, we were surprised not to find evidence for a preference for the familiar habitats offered visually. We continued with bigger sample sizes and an improved experimental design, and a comparison between adult- (>2 years old at the time of testing) and immature knots (< 2 year old at testing) was added. Immature birds do not breed in the first summer after their birth and most do not physiologically prepare for northward migration (e.g. Verhoeven et al. 2016). On this basis we expected adult, but not immature, knots to show a seasonal change in habitat preference.

Methods

This experiment included 19 adult and 8 immature knots. This time, to encourage natural circannual phenotypic rhythms to occur, all birds were continuously held in outdoor aviaries where they were exposed to ambient temperature and photoperiod (Karagicheva et al. 2016). Focal birds were only moved to the experimental room immediately prior to testing. In Preference Test I, the pre-breeding testing period took place very close to the known peak departure in the second week of May (Swennen 1992). In Preference Test II we therefore advanced the pre-breeding trials in time. The start date for the pre-breeding season remained the same, hence prolonging the time between two test periods. We carried out 16 trials per bird from 9 March to 17 April 2015 (39 days) and again from 19 May to 16 June (27 days). A bird was never tested more than once a day. This resulted in 16 unique choices per bird per season, 32 choices per bird in total. Further refining Preference Test I, to test for preference for a habitat type (mudflat or tundra), rather than for one specific picture of a habitat, this time a novel combination of pictures was presented each trial. Thus, 16

different pictures of mudflat- and tundra landscape were used in random combinations (Figure 3).

In Discrimination Test I and Preference Test I there was no physical barrier between the screens, so that knots could easily switch position. As knots have a viewing angle in both eyes that is bigger than 140° (Martin and Piersma 2009), they can assess the two images simultaneously and there is no need for birds to make a dichotomous choice. To force knots to make an unambiguous decision, we redesigned the room, the pictures now being projected side by side on one wall of the indoor room rather than opposite each other (by means of higher resolution LCD projectors, properties; type: panasonic-pt-vx600e, SVGA: 800 x 600, light intensity: 5500 lumen, contrast: 10 000 : 1, mass: 4.8 kg). The two 2.5 m high and 3.5 m wide projection screens were separated by a black plastic tarp dividing the room in two halves, with the tarp leading right up to the release cage (Figure 3). Consequently, once a bird had left the release cage, the choice would be irreversible. Note that during training (from 20 February to 7 March 2015) the mid-room tarp ended one meter in front of the release box. This enabled the birds to see both screens and gave enough space for birds to fly around the division after leaving the release box. During the actual tests the division was pulled up to the release cage to enforce an early an unambiguous choice. No food was offered in the experimental room during training and testing. Also, the water level in the aviary was raised so that the birds had to fly 5 m from the release box to reach a sandy patch (1 m x 2 m) in front of one of the screened images (see Figure 3).

In the first five minutes of testing the bird was held in the release cage with the screened panel closed. During this time we recorded the time that the bird was on either side of the release cage, i.e. facing and possibly looking at either one of the two projected images. The release cage was remotely opened to allow the bird to indicate its preference by landing on one of the patches. In case a bird would not leave the

release cage within 10 minutes, the release cage was slowly lifted (by means of another pulley mechanism) so that the bird was forced out and indicate its picture of choice. Again, ten minutes after leaving the release cage a trial ended.

The analyses were identical to the analyses for Preference Test I. However, to examine any difference in preference between adults and immatures, a fixed effect for age was added (0 = adult, 1 = immature). Contrary to Preference Test I, daily plumage scores, recorded before each test, were used rather than interpolated scores.

As birds in the release cage were in the position to observe the images during the five minutes before the front panel was opened, we considered the possibility that the birds already give away their habitat preference by spending more time facing the image of preference. To test for this, we ran a similar model, using a bivariate response variable based on the percentage of time that the bird spent on either site of the release cage in the five minutes before the panel was opened. In this analysis, the same fixed- and random effects were included, but the response variable was replaced by a response variable indicating if the birds spent > 50% of its time in the release cage facing the tundra image (1) or the mudflat image (0).
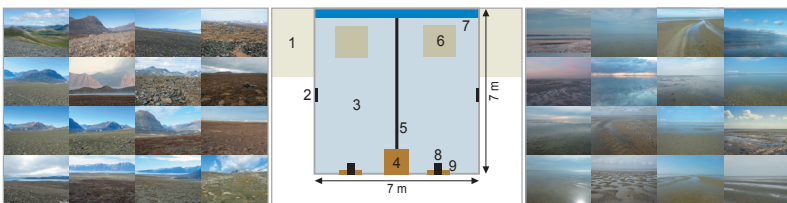
Figure 3. Experimental set-up for Preference Test II. From left to right: 16 unique pictures representing breeding ground (known red knot habitat at the high arctic tundra in Greenland), the experimental shorebird facility: upper left; projection screen south, upper right; projection screen north, (1) aviary, (2) camera, (3) submerged mud-flat (high-tide), (4) release cage, (5) division, (6) sandy patch, (7) projection screen, (8) projector, (9) entrance door, 16 unique pictures representing wintering ground (known red knot habitat in the Dutch Wadden Sea). During the experiments only one image representing tundra and mudflat was presented and the projection side was designed to vary randomly.

Results

Despite what we thought were experimental improvements, the results of Preference Test II were very similar to Preference Test I. The knots showed no preference, and no seasonal change (all 95% CI overlap with 0; Table 2, Figure 6). There was no association between habitat preference and the extent of breeding plumage (Table 2). There was no bias towards one side of the experimental room and no variation in preference among individuals (Table 2, Appendix S1 Table S3). Furthermore, there was no autocorrelation between successive trials (two-sided Runs Test: $Z = 0.80$, $p = 0.42$, Appendix S1 Table S3). Notably, there was no difference in habitat preference between immature and adult knots (Table 2, Figure 6, Appendix S1 Table S3).

The analyses based on the time facing either of the two images during the five minutes before the front panel was opened, revealed that the birds actually spent more time in the half of the release cage exposed to the Tundra image during the breeding season than during the pre-breeding season (95% CI of the intercept does not overlap with 0) (Appendix S1 Table S4). During the pre-breeding season birds spent less time facing the Tundra image, but this difference was not significantly different from zero (the 95% CI for Season overlaps with 0). We found no association between time facing the Tundra image with breeding plumage, projection side or age (all 95% CI broadly overlap with 0, Appendix S1 Table S4).

Discrimination Test II

Since we found no distinct preference for habitat in Preference Test II, we once more wanted to confirm that knots are able to distinguish projected images using what we thought was an improved experimental design and protocol.

Methods

The experimental layout and procedure was identical to Preference Test II. However, to reduce the stimulus complexity in this experiment natural pictures were replaced by images made in Microsoft Powerpoint and represented a very simplistic graphical visualization of mudflat or tundra. Whereas the mudflat image showed a horizontal edge to a brown surface (mimicking a horizon), the forest image showed a curvy green area. Both images contained equal amounts of blue (sky) (Figure 4). For this experiment six birds were assigned to the 'mudflat' group and trained to find food in the feeder near the mudflat image. The other six knots were assigned to the 'forest' group and trained to find food near the forest image (Figure 4).

Having taken part in Preference test II, the knots were already acquainted with the experimental room and general testing procedures. The training on the images took place in two series of training between 17 and 24 September 2015. In the first four days of training each group of six birds was introduced to the room twice a day with the image to be required on one side, and a dark (black) screen on the other side. On both sandy patches and in front of the screens a feeder was placed. Only the feeder in front of the image contained trout food pellets, in front of the black screen the feeder was empty. During training the birds received no other food than what they could eat at the feeders. When body mass dropped below 100 g, additional trout pellets were offered to this bird outside the training sessions. After training in groups, training continued with a period of four days in which knots were introduced to the room twice a day solely. During the training period the birds received no other food than what they could eat at the feeders.

Actual testing took place from 25 to 29 September 2015. Knots were individually tested in a random order twice a day (n = 8), having to make a choice between the familiar and the non-familiar images. Contrary to

Discrimination Test I, no reward was offered during testing. Instead, the knots received food during the rest of the day. When the release cage was opened, the bird indicated its image of choice by flying to the projection, or rather the patch in front. Again, when a bird would not leave the release cage within 10 minutes, the bird was forced to make a choice. A trial ended 10 minutes after the choice was made.
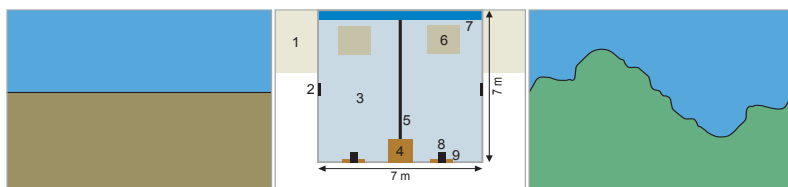
Figure 4. Experimental set-up for Discrimination Test II. Left; graphical representation of mudflat, Right; graphical representation of tundra; the experimental shorebird facility: (1) aviary, (2) camera, (3) submerged mudflat (high-tide), (4) release cage, (5) division, (6) sandy patch, (7) projection screen, (8) projector, (9) entrance door. During the experiments the projection side was designed to vary randomly.

Results

The knots, which incidentally did not show any seasonal change in preference in this experimental set-up, did actually distinguish between the projected images (after back transformation from the intercept model: β = 61%, 95% CI = 50%, 69%, Figure 5). Like in Preference Test I the birds trained on the forest image again performed better than the birds trained on the mudflat image (Appendix S1 Table S5, Table 1; p = 0.03). And again the knots showed bias to one of the sides of the room, this time preferring the north over the south side (Table 1). Repeated testing did not affect choice (Appendix S1 Table S5; one-sided Runs Test: Z = -0.70, p = 0.24).

Table 1. Log-odds for the focal bird to choose the rewarded image in the experiments on Discrimination Tests I (left) and II (right).

	Discrimination Test I (n = 4)	Discrimination Test II (n = 12)
Fixed effects	β (95% CI min, max)	β (95% CI min, max)
Intercept[a]	0.95 (0.61, 1.45)	-0.15 (-0.83, 0.64)
Projection Side (north)	**-0.54 (-0.89, -0.01)**	**1.69 (0.91, 2.78)**
Group (mudflat)	**-0.48 (-0.96, -0.01)**	-0.61 (-1.76, 0.06)
Random effect	σ (95% CI min, max)	σ (95% CI min, max)
Bird ID	**0.03 (0.00, 0.09)**	**0.04 (0.01, 0.09)**

Note: The logit transformed binary response choice (1 = correct, 0 = false) was based on the first feeder that was visited by the focal knot. Significant effects (i.e., whose 95% CI do not overlap with 0) are indicated in bold. (a) The intercept represents the choice made by an individual of the group trained on the forest image with the correct picture being projected on the south side of the testing arena.

Table 2. Log-odds for the focal bird to choose the projection representing tundra lanscape during the experiments of Preference Test I (left) and Preference Test II (right).

	Preference Test I (n = 13)	Preference Test II (n = 27)
Fixed effects	β (95% CI min, max)	β (95% CI min, max)
Intercept[a]	-0.13 (-0.72, 0.37)	0.17 (-0.08, 0.46)
Season (Pre-breeding-)	-0.02 (-0.51, 0.58)	0.06 (-0.27, 0.48)
Plumage (Winter-)	0.11 (-0.61, 0.50)	-0.37 (-0.64, 0.23)
Age (immature)	NA	0.03 (-0.27, 0.41)
Projection Side (North)	-0.09 (-0.57, 0.51)	-0.04 (-0.35, 0.16)
Random effect	σ (95% CI min, max)	σ (95% CI min, max)
Bird ID	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)

Note:The logit transformed binary response choice (1 = tundra, 0 = mudflat) was based on the first feeder that was visited by the focal knot. (a) The intercept represents the choice made by a mature individual in summer plumage, during the breeding season with the correct picture being projected on the south side of the testing arena.

General Discussion

By means of two tests we were able to show that knots can memorize and distinguish projected images of landscapes. Nonetheless, we were unable to find evidence for a vision-based preference for habitat which changes seasonally. We do not interpret our inability to demonstrate a (changing) visual preference as a strong rejection of the idea that knots may have a seasonally changing preference for habitat. As we will argue, we believe that the testing protocols used may have been an oversimplification of the natural world and the way knots use visual cues.
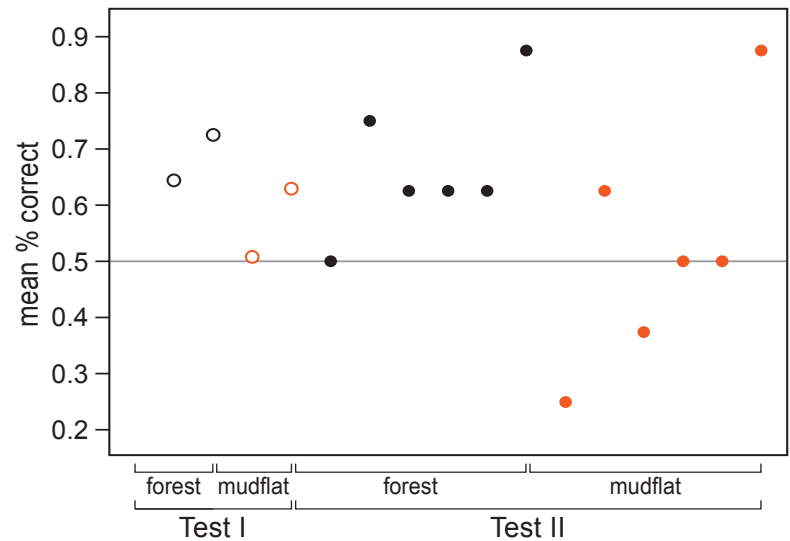
Figure 5. Individual performance during Discrimination Tests I and II. Dots represent mean values for correct choices per knot assigned to the forest- or the mudflat group for Discrimination Test I (open circles) and Test II (closed circles). The horizontal line represents 50% correct or random choice.

Discrimination I and II

The two experiments on image discrimination revealed that knots can learn to discriminate between projected images (Figure 5). The fact that the mean scores in both tests remained relatively low (64% and 61% correct) may well be due to the inquisitive nature of knots. In previous

operant conditioning experiments on the sensory modalities of their bill tip (Piersma et al. 1998), knots kept exploring the alternatives, even when fully informed. Contrary to expectation the ability to discriminate was lower in test II when compared to test I. In test II we attempted to reduce the stimulus complexity in order to emphasize the differences, and potentially simplify discrimination, between contrasting images. However replacing photographs of natural landscapes with simplistic line drawings did not result in better discriminative abilities in the knots. Stimuli complexity is in the eye of the beholder; while intuitively one may think that abstract line drawings may be easier to discriminate when compared to more complex, stimuli rich pictures, notably pictures, may be easier to discriminate due to their similarity to the natural scenes they represent (Fetterman 1996).

In both discrimination tests, but not in the preference tests, the knots favoured one side of the room over the other, albeit in opposite directions. We suggest that this may be due to noise from outside the room, especially during Discrimination Test I when the birds were not only tested, but also housed in the room. Regardless of the side bias, and for reasons that are unclear, the knots trained on the forest images performed better than birds trained on the images of mudflat.

The ability to memorize and discriminate between pictures has been studied in many species including spiders, reptiles, fish and mammals (see Bovet and Vauclair 2000 for an overview). Most experiments about picture recognition in birds were done with pigeons. The duration of training and the required number of tests in both Discrimination Test I and II to reach significant results was relatively low compared to other studies on picture recognition in pigeons (Wilkie et al. 1989, Dawkins et al. 1996, Lechelt and Spetch 1997, Aust and Huber 2006, Spetch and Friedman 2006). However, episodic memory ('what-where-when') is also important to achieve homing, and occurs in brood parasitism and food caching behaviour (Emery and Clayton 2004). Birds have been

shown to be able to recover information about flock mates and cached food items years after the initial experience (Emery 2016).

Although cognitive experiments in shorebirds are scarce, field observations of birds returning yearly to the same locations (Lok et al. 2011, Verhoeven et al. 2019) to pair up with the same partner (Kentie et al. 2014), are clear indications that shorebirds possess long-term memory. In an exceptional experiment with turnstones (*Arenaria interpres*), Whitfield (1986 1987) showed that individuals are able to remember and recognize conspecifics based on their plumage characteristics. For knots, direct evidence for memory is anecdotal. 'Peter' was a knot of the *islandica* subspecies kept as a pet for 20 years (see Piersma 2002 for details). It remembered and recognized the barking sound of the pet dog he used to live with even when the barks were played back to him 10 years after the dog died (TP pers. obs.). In this example, a red knot showed evidence of long-term memory for audial information. The length of time over which knots can remember visual cues remains to be established. The testing protocol described here gives us one way to its assessment.
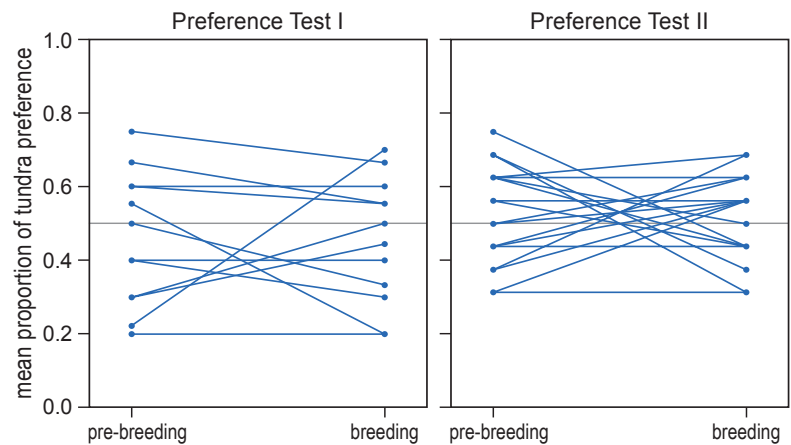
Figure 6. Individual changes in habitat preference. Mean preference values for tundra landscape (dots) are connected for each individual between the Pre-breeding and breeding season. The horizontal line indicates 50% preference for tundra, or random choice. A seasonally appropriate change in habitat preference would have resulted in a positive slope between means for each individual.

Preference tests I and II

We were not able to demonstrate a preference for either of the two types of landscape pictures (mudflat vs. tundra). Indeed, one possibility is that knots do not show seasonally changing preferences for habitat. Instead seasonally migration may be motivated by means of evolved endogenous phenological mechanisms related to reproduction that are unrelated to habitat.

Alternatively, the knots learned, in successive trials, that the projected landscapes were not 'real'. In fact, during the first few trials we observed birds flying into the part of the projection screen taken up by the sky, suggesting that they took this part of the image 'seriously'. Having learned the artificial nature of the image, the birds may have lost interest. Interestingly, when we only consider the first trial in which knots were exposed to natural images representing mudflat and tundra in the pre-breeding season, we found a mean preference of 82% for mudflat in Preference Test I. During Preference Test II knots were already exposed to natural images of mudflat and tundra during training, in the first training session they showed a mean preference for mudflat of 57%. Limiting our dataset to only the first trial reduces the possibility to test for individual changes in habitat preference from one season to another. To prevent this potential learning effect, we suggest that for future experiments many birds should be tested once and in different seasons, rather than repeatedly.

Our inability to demonstrate a preference for either of the two types of landscape pictures may also be due to the testing protocol. When we analysed the position of birds in the release cage in Preference Test II, we found that during the breeding season the knots spent more time looking at tundra images than expected under the assumption of random choice. This suggests that a nuanced scoring method, maybe based on time looking at an image, rather than flying to and landing on a patch in front of it, would lead to more explicit results.

Still, the incomplete development of breeding plumages in some individuals (Appendix S2 Figure S1 and S2) could also indicate that some or all knots never reached the physiological threshold at which they would experience the motivation to take-off for the tundra breeding grounds (see Piersma and Jukema 1993, Piersma et al. 2000). Free-ranging knots in the Wadden Sea are known to refrain from spring migration when they are not physiologically fit (TP pers. obs.). However, incomplete physiological preparation does not explain why knots did not show preference for the mudflat images during the non-breeding season.

As for all experiments regarding natural responses in wild animals that are tested in artificial settings, we cannot exclude the possibility that the complete lack of preference followed from a general state of unhappiness related to the captive conditions (Howard 1952, Wyers 1994). Note that the results of Discrimination Tests I and II show that the same knots tested under similar conditions were able to acquire a preference for images when a reward was offered. Nevertheless, Discrimination Tests I and II also revealed that individuals vary in their ability to memorize and recognize projected images. Similarly, it is possible that knots vary in their ability to memorize and recognize natural habitats, for example because they differ in experience (Pravosudov et al. 2006, Berbert and Fagan 2012) or social status (Gibson and Kamil 2009). Unfortunately we do not have the power to find among individual differences in performance on the log-odds ratio of choosing the right images.

We propose that the visual representations of landscapes presented here fell short. This could be due to optical limitations of the projected images, including the angle at which the pictures were taken, the absence of depth (note that the overlap of both fields of vision should enable knots to perceive depth; Martin and Piersma 2009), and the probable varying reception of colours when compared to humans,

most obvious in the ability to see UV-light (Cuthill 2006). Although several species of birds are found to be capable of object-to-picture transformation in the laboratory (Watanabe 1997, Spetch et al. 2006, Spetch and Friedman 2006), results of object-to-picture transformation regarding real world locations are ambivalent (see Wilkie et al. 1989 for an example of successful picture-to-location transformation and Dawkins et al. 1996 for an example in which pigeons failed to translate pictures to real locations).

If purposive activities like (breeding-) habitat choice would only be provoked if an integration of multiple, specific sensory demands comes together (Lashley 1938), knots may need more than visual cues to recognize habitats. We suggest that in future experiments on habitat preference, other sensory cues such as smell (Wallraff 1990, Nevitt 2008, Wikelski et al. 2015), sound (Hagstrum 2000) and/or magnetic field (Alerstam 1990a) should be included. In this study we were not able to confirm that knots show preference for seasonally appropriate habitats. When taking all above mentioned limitations (the absence of other natural cues, definition of preference, timing, individual variation etc.) into account, we consider the absence of a seasonally changing habitat preference in our tests as the result of too much experimental simplification, typical of behavioural experiments in laboratory settings (Wyers 1994). Consequently, we hope that this detailed account of what we managed to achieve will inspires others, just as we have been inspired by the work of Roberts and Weigl (1984). Whereas scores of studies address questions on 'how' birds migrate (in terms of physiology, energy budgets and navigation) and 'why' birds migrate (fitness consequences), questions on the causation of migratory behaviour (Hogan 2017) are sparse (Piersma 2018). The rarity of studies on cognitive processes in wild birds will indicate fashions in science, but may also reflect the serious methodological challenges that come with it. With this study, touching upon the role of memory during migration, we hope to have started to scratch the

surface on the role of cognitive mechanisms motivating seasonal migration (Hogan 2017).

Authorship Statement

EMAK, JH and TP conceived and designed of the experiments. EMAK collected and analysed the data. EMAK wrote the manuscript with help from TP and JH. All authors gave final approval for publication.

Compliance with ethical standards

Ethical Approval: This work was carried out under auspices of the Animal Experiment Committee (DEC) of the Dutch Royal Academy of Sciences (KNAW) (permit DEC-NIOZ 10.05). Conflict of Interest: The authors declare that they have no conflict of interest.

Data availability statement

The full datasets and reproducible code are available from EMAK upon request.

Acknowledgements

Electronic supplementary material

Appendix S1. Discrimination Tests and Preference Tests

Table S1: Discrimination Test I. Mean percentage of replicates correct per bird (nBird = 4). Number of no response trials in parentheses.

Birdid/trailnr	1-22	23-44	45-66	67-86	Total
LR (mudflat)	0.56(6)	0.58(3)	0.40(2)	0.50(8)	0.52
NN (mudflat)	0.67(6)	0.57(1)	0.65(2)	0.68(1)	0.64
YG (forest)	0.60(2)	0.68 (0)	0.81(1)	0.82(3)	0.73
YL (forest)	0.70(1)	0.55(2)	0.65(2)	0.70(0)	0.65
Total	0.63	0.60	0.63	0.68	

Table S2. Preference Test I. Mean percentage preference for tundra per individual (n =13) for the pre-breeding and breeding season. Number of missing values per bird per season are given in parentheses. Percentages in bold indicate the birds that showed the predicted change in habitat preference.

birdid/SEASON	Pre-breeding	breeding
GG	0.55 (1)	0.67 (7)
GL	0.44 (1)	0.30 (0)
GN	0.70 (0)	0.22 (1)
GR	0.50 (0)	0.30 (0)
LG	0.33 (1)	0.50 (0)
NG	0.56 (1)	0.60 (0)
NL	0.30 (0)	0.40 (0)
NR	0.60 (0)	0.60 (0)
RG	0.40 (0)	0.40 (5)
RL	**0.20 (0)**	**0.56 (1)**
RN	0.20 (0)	0.20 (5)
RR	0.50 (0)	0.50 (0)
YN	0.67 (7)	0.75 (6)
overall	0.45 (11)	0.45 (25)

Table S3. Preference Test II. Mean percentage preference for tundra per individual for the pre-breeding and breeding season. All individuals (n=27) were tested in 32 trials. Percentages in bold indicate the birds that showed the predicted change in habitat preference.

	age at capture	preferred habitat	
		pre-breeding	breeding
G	immature	**0.44**	**0.56**
N	immature	0.56	0.38
new	immature	0.69	0.38
P	immature	0.56	0.44
PG	immature	0.56	0.56
R	immature	0.50	0.50
RY	immature	**0.44**	**0.63**
Y	immature	0.63	0.50
N4PNYN	mature	0.50	0.50
N4PYRP	mature	0.56	0.50
N8GNGR	mature	**0.38**	**0.69**
N8GNNR	mature	0.44	0.44
N8GRGN	mature	0.63	0.44
N8GRYG	mature	0.63	0.50
N8GYGN	mature	0.50	0.50
N4PNYP	mature	0.50	0.63
N4PNYY	mature	0.69	0.63
N4PYGN	mature	0.63	0.50
N4PYNG	mature	**0.44**	**0.75**
N4PYNY	mature	0.56	0.38
N4PYPY	mature	**0.38**	**0.69**
N4PYRN	mature	0.63	0.63
N4PYRR	mature	0.50	0.50
N8GRGR	mature	0.56	0.31
N8GRNN	mature	**0.31**	**0.69**
N8GRRN	mature	0.50	0.63
N8GRYN	mature	0.31	0.31
overall mean		0.52	0.52
immature (n = 8)		0.55	0.49
mature (n= 19)		0.51	0.54

Table S4. Preference Test II.

	Original Preference Test II (n = 27)	Time looking at Tundra image before release (n = 27)
Fixed effects	β (95% CI min, max)	β (95% CI min, max)
Intercept[1]	0.17 (-0.08, 0.46)	**0.26 (0.03, 0.57)**
Season (Pre-breeding-)	0.06 (-0.27, 0.48)	-0.15 (-0.48, 0.28)
Plumage (Winter-)	-0.37 (-0.64, 0.23)	-0.24 (-0.52, 0.36)
Age (immature)	0.03 (-0.27, 0.41)	-0.12 (-0.41, 0.27)
Projection Side (North)	-0.04 (-0.35, 0.16)	-0.05 (-0.36, 0.15)
Random effect	σ (95% CI min, max)	σ (95% CI min, max)
Bird ID	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)

Note: Log-odds for the focal bird to choose the projection representing tundra landscape during the experiments of Preference Test II (left) and a test with a binary response variable based on the percentage of time looking at the Tundra images before the opening of the front panel (1 = tundra, 0 = mudflat). Significant effects (i.e., whose 95% CI do not overlap with 0) are indicated in bold. [1]The intercept represents the choice made by a mature individual in summer plumage, during the breeding season with the correct picture being projected on the south side of the testing arena.

Table S5. Discrimination Test II. Mean percentage of correct choices per replicate (nBird = 12). A response was provoked for every replicate.

β correct/replicate	Overall	1	2	3	4	5	6	7	8
forest	0.67	0.83	0.67	0.67	0.83	0.83	0.50	0.50	0.50
mudflat	0.52	0.67	0.17	0.50	0.67	0.67	0.67	0.17	0.67
overall	0.59	0.75	0.42	0.58	0.75	0.75	0.58	0.33	0.58

Appendix S2: Plumage scores
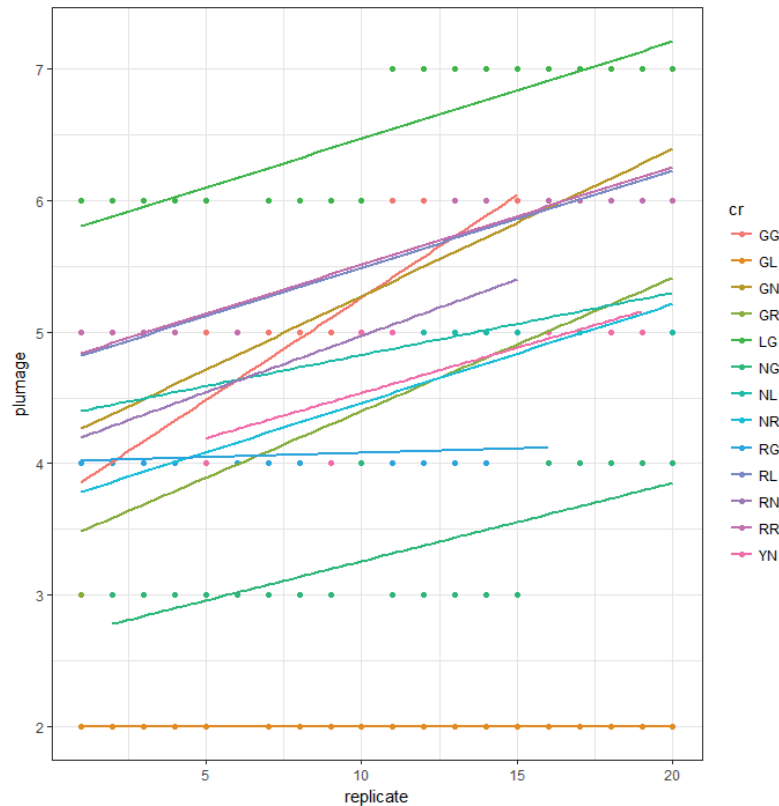
Figure S1. Plumage scores in Preference Test I. The y-axis indicates the breeding plumage score (1-7 from 1 = full winter plumage to 7 = full breeding plumage) for each knot in *Preference Test I*. Plumage scores were done once a week and values for intermediate days were interpolated. The x-axis represents each behavioural observation with replicate 1-10 representing pre-breeding, and 11-20 the breeding season. In the analysis scores 1-4 are considered non-breeding plumage and 5-7 are considered breeding plumage.
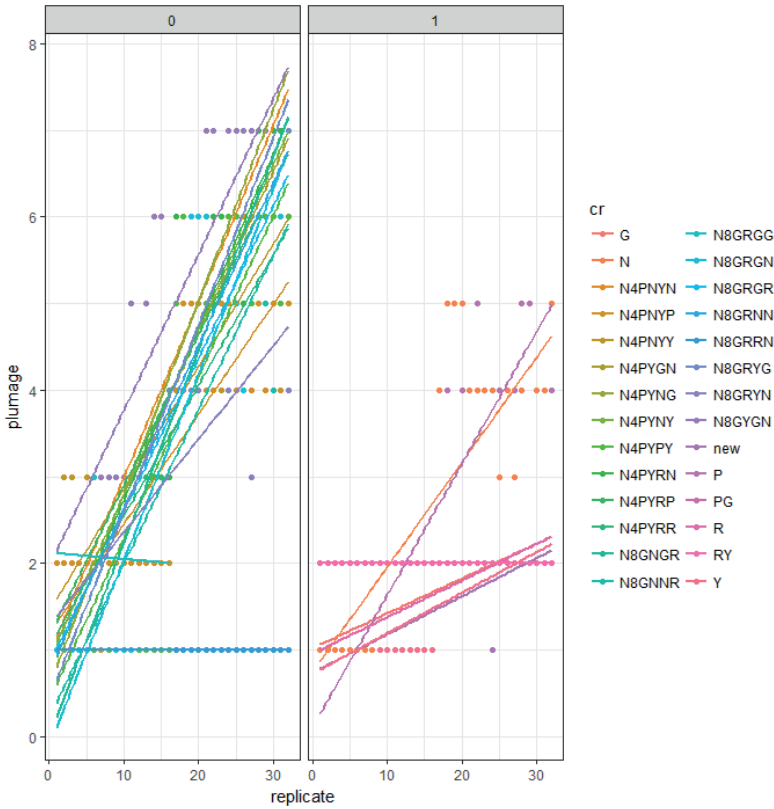
Figure S2. Plumage scores in Preference Test II. The y-axis indicates the breeding plumage score (1-7 from 1 = full winter plumage to 7 = full breeding plumage) for each knot in *Preference Test II*. Plumage scores represent raw data and were taken immediately after every behavioural observation. The x-axis represents each behavioural observation with replicate 1-16 representing pre-breeding, and 17-32 the breeding season. The left panel shows the individual scores for the mature knots, that are expected to breed, the right panel shows the plumage scores for the immature individuals, that are not expected to breed in their first winter. In the analysis scores 1-4 are considered non-breeding plumage and 5-7 are considered breeding plumage.

7

CHAPTER 7

KNOTS AS INDIVIDUALS

Eva M.A. Kok

Thesis outline

In the writing of this thesis I experienced that the categorization of relevant questions according to Hogan's conceptual framework (Chapter 1; Table 1) provided me with a way of thinking about the different layers of complexity that underlie migratory behaviour. In the process, I discovered that different kinds of questions require different kinds of methodological approaches. I will now discuss how the use of standardized behavioural assays in combination with high definition tracking of free-living individuals may help scientists to make progress in all nine of Hogan's aspects for studies on individual variation in migratory behaviour.

First, I will elaborate on how standardized behavioural assays can aid in studying the causes and consequences of behavioural variation. Second, given the uncertainties about the co-variance between behaviours measured in highly standardized environments and the equivalent movements as free-ranging individuals, I explore alternative methods to study the development of individual behaviour in free living individuals. Finally, I discuss implications of the findings in this thesis and make some suggestions for future research in red knots.

Standardized assays

Use of standardized assays

In the second part of this thesis I used standardized behavioural assays to study potential causes of among individual differences in behaviour. Individuals are said to differ consistently from one and another (i.e. the expression of the trait is repeatable) when a portion of the total variation present in the population can be attributed to variation expressed among individuals rather than within (Sih et al. 2004, Bell et al. 2009, Réale et al. 2010a). Therefore, to allow for the calculation of consistent among individual differences in

behaviour, the behaviour has to be measured repeatedly under similar environmental conditions and in multiple individuals (Sih et al. 2004). By means of controlled manipulations of testing conditions, the use of standardized behavioural assays enables researchers to study the variation that exists at the level of the individual as well as at the level of the population, but also to address questions related to causes and consequences of behaviour. The controlled introduction of variation in the expression of individual traits enables us to separate changes in among- and within individual (co-)variation between labile traits (Chapter 4) and reveal differences in timing of the integration of traits during ontogeny (Chapter 5) that could never have been revealed in free-living individuals.

Considerations for the use of standardized assays

For practical reasons, experiments including repeated measures of multiple individuals under controlled conditions are often carried out in captivity, rather than in the wild on free-living individuals (e.g. Sinn et al. 2008, Exnerova et al. 2010, Kurvers et al. 2010, David et al. 2012, Tremmel and Müller 2013, Schuster et al. 2017). As a result, there is a lot of debate on how results obtained in standardized assays have to be interpreted for free-living individuals (Archard and Braithwaite 2010, Carter et al. 2013, Niemelä and Dingemanse 2014, Fisher et al. 2015). Evidence is accumulating that consistent among individual differences in behavioural traits obtained in standardized assays correlate with free-ranging (dispersal-) movements in many species, including insects (Odermatt et al. 2017, Dällenbach et al. 2018), fish (Wilson 1998, Jones and Godin 2010, Chapman et al. 2011a, Myles-Gonzalez et al. 2015, Bierbach et al. 2017, Hirsch et al. 2017, Hoch et al. 2019), birds (Dingemanse et al. 2003, Minderman et al. 2010, David et al. 2012, Liebl and Martin 2012, Wuerz and Krüger 2015, Nilsson et al. 2016, Morganti et al. 2017, Dale et al. 2019, Krüger et al. 2019), mammals (Sussman and Ha 2011, Debeffe et al. 2014, Found and St. Clair 2016, Schuster et al. 2017), arachnids (Bosco et al. 2017) and reptiles (Sakai

2018). Also in red knots, consistent among individual differences in exploration, measured in an experimental arena during temporary captivity, predicted the probability for a bird to be locally resighted after release (Bijleveld et al. 2014). Interestingly, we were not able to replicate this result. We found no evidence for a correlation between the resighting rate of individuals after release and the previously obtained exploration scores (see Chapter 5; mean exploration scores for the group of knots that were locally resighted $\mu= 0.27$, 95% CI = 0.22, 0.32, n = 39, and for the group that was not $\mu= 0.28$, 95% CI = 0.17, 0.40, n = 27). Possibly variation in free ranging movement does not exclusively correlate with variations in behavioural phenotype measured in our standardized behavioural assays because of interactions with the ecological context that we have removed in the highly controlled conditions of our test. The absence of this correlation is unfortunate because if the correlation persisted, it would have given us a simple yet powerful tool to quantify large scale movement behaviour.

In fact, similar discrepancies have been seen in other species. In a study on great tits (*Parus major*), fast exploring individuals readily visited novel feeders indicated by a conspecific, while slow explorers did not (Marchetti and Drent 2000). However, at the same time, slow exploring great tits were more inclined than fast explorers to independently extend their foraging territory. Earlier studies also report inconclusive results on the relationships between outcomes of standardized behavioural assays and free-ranging movements in relation to habitat (Harrison et al. 2017, Santicchiaa et al. 2018), seasonal variation (van Overveld et al. 2014), resource availability (Wilson 1998, Marchetti and Drent 2000, Exnerova et al. 2010, Toscano et al. 2016, Krüger et al. 2019, van der Kolk et al. 2019), the presence of conspecifics (Webster and Ward 2011, McCowan et al. 2015) and the presence of predators (Cote et al. 2013).

Standardized behavioural assays are especially valuable when

addressing questions about the causes and consequences of individual variation (Chapter 4-6 this thesis). However, because experienced environmental conditions play a key role in developing and maintaining among individual variation (see results Chapter 5), discrepancies in the direction and strength of behavioral co-variance structures between outcomes of standardized behavioural assays and free-ranging movements are actually expected. Clearly, natural variation in migratory phenotypes present in a wild population of migrants can best be studied in the wild.

Implications of these findings and outlook to the future

Development of natural behaviour

For new studies on the development of natural variation in behaviour I suggest to prioritize the development of methods to study current variation in behaviour in the wild. Such assays will necessarily need to account for natural environmental variation. Future research on individual variation in exploration behaviour in knots should include high definition tracking of individual variation in behaviour while taking environmental variation (resource landscape, conspecifics and predators) into account. Excitingly, this is what Selin Ersoy and Allert Bijleveld currently at NIOZ have set out to do. In addition, a comparison of birds belonging to different age groups may shed light on the role of experience (i.e. age-related variation) in the development of behavioural variation.

Development of migratory behaviour

Because the temporal and spatial variation in the flexibility of the development of individual migratory routines has important implications for the ecology and the science-based conservation of species and their habitats, it is important to study migrations from a

very young age onward. With the development of robust miniature satellite-linked tracking devices fueled by solar energy, in combination with a durable and noninvasive attachment methods (Rappole 1978, Chan et al 2016, Box 1 this thesis), it is now feasible to track an individual over the entire lifetime. In theory this would enable researchers to visualize age-related variation in migratory routines between experienced and inexperienced individuals and compare the changes in individual migratory behaviour over time.

Understandably, because of the price of the tags and the fact that survival is rather low in the most interesting developmental period after independence, up to now studies on the development of migratory routines with decent sample sizes (n > 10 individuals) can be counted on one hand (see Chernetsov et al. 2004, Meyburg et al. 2017, Vansteelant et al. 2017, Verhoeven and Loonstra et al. in prep.). The launch of the novel German-Russian satellite-linked tracking system specifically designed for the use in studies on (bird) migration (ICARUS, International Cooperation for Animal Research Using Space, https://www.icarus.mpg.de/en), in the near future may therefore critically help this field when the tracking of many individuals from hatching onwards becomes much more affordable.

The development of individual migratory routines in red knots seems to be molded by experiences during ontogeny (see Box 2). Given that spatial distribution of individual red knots of the *C. c. canutus* and *C. c. islandica* subspecies can be explained by the resource distribution at their subspecies-specific non-breeding grounds (Oudman et al. 2018) suggests, that knots may be able to adjust their behaviour to the environment. Whether this is the result of a phylogenetic process that selected for the best adjusted individuals or if such an adjustment results from individual adaptations and learning in juveniles when they first arrive at their southerly non-breeding grounds, can now, at least in principle, be studied by means of multi-year tracking of juveniles

belonging to populations that face rapid environmental changes (such as the *C. c. piersmai* knots in the Yellow Sea, Chapter 3).

Conclusion

What distinguishes within individual canalization of behaviour described here, from (evolved) general variation in phenotypic flexibility (Piersma and Drent 2003, Piersma and van Gils 2011), is that the canalization of the expression of behaviour within an individual does not necessarily lead to a reduction in flexibility. It can therefore be compared with learning; it is a developmental cognitive process. Research on the cognitive causes of migratory behaviour are rare (Hogan 2017, Piersma 2018, but see Chapter 7 this thesis), perhaps because cognitive work in animals is frequently considered non-scientific (Darwin 1872, de Waal 2016). I would suggest that it is also because the black box of the minds (of animals) appears impossible to open. If one's prior assumption is that animal are little machine, with no emotions, feelings or minds, the formulation of research questions will be restricted by this opinion (*sensu* Howard; Howard 1952, Meijer 2019). A structural classification of question such as the one proposed by Hogan (see Chapter 1, Table 1) may guide biologists to cover all aspects of avian migration, including cognitive phenomena, and aid in the formulation of a more holistic description of bird migration.

Acknowledgments

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Theunis Piersma*, **Eva M.A. Kok***, Chris J. Hassell, He-Bo Peng, Yvonne I. Verkuil, Guangchun Lei, Julia Karagicheva, Eldar Rakhimberdiev, Paul A. Howey, T. Lee Tibbitts, and Ying-Chi Chan. When a typical jumper skips: itineraries and staging habitats used by red knots *Calidris canutus piersmai* migrating between northwest Australia and the New Siberian Islands (in prep. for Ibis). * authors contributed equally

Summary

Red knots (*Calidris canutus*) are shorebirds that, together with many other animals, migrate yearly to and from their northern breeding grounds. As part of the migratory routine red knots (hereafter: knots) yearly undergo a remodeling of their body to be physiologically prepared for the long flight and following reproductive season. Since the artic summers are short, timing is of the essence. So are the circumstances encountered en route, such as the use of stopovers and the path to follow. Although the process of migration seems very complex and particularly susceptible to environmental influences previous work has shown that many experienced migrants are very consistent in their migratory routines.

The aim of this thesis is to increase understanding of the development of individual migratory routines. In the first part of this thesis, before getting to the development of variation in migratory behaviour, I first explore individual variation in migratory behaviour present in wild knots (**Chapters 2** and **3** and **Box I**). **Chapter 2** and **3** are the first detailed accounts of the migratory routines of knots belonging to the *C. c. islandica* and *C. c. piersmai* subspecies using a novel 2 g solar-powered satellite transmitter attached to the knots by means of a durable, tailor-made backpack harness (see **Box 1**). In **Chapter 2** we visualized the flight paths travelled by a female red knot (*C. c. islandica*) during two northward and two southward migrations and suggest that red knots can migrate across the Greenland Icecap along the shortest-, orthodrome-like path while being able to adjust their course in response to local conditions. In **Chapter 3** we show that knots of the *C. c. piersmai* subspecies, unlike the other five *C. canutus* subspecies, make several stops during northward migration. This pattern of visiting multiple staging areas before reaching the Yellow Sea suggests the availability of suitable staging sites along the Chinese coast.

Observing variation in migratory routines does not necessarily tell us about the causes of the observed variation. In the second part of this thesis I used standardized assays and knots temporarily held in captivity, to study potential (developmental-) causes of among individual differences in trait expression. In **Chapter 4** and **5** I present the results of a longitudinal study spanning two consecutive years during which we examined the (age-related) variation in certain physiological and behavioural traits in response to changes in diet. In **Chapter 4**, we found that individuals adjust their pectoral muscle mass (i.e. flight capacity) to changes in gizzard mass (i.e. digestive capacity), suggesting that the individual trade of in muscle mass between the two organs has evolved as a result of a migratory lifestyle (see **Chapter 4**). Using the same set of birds as in **Chapter 4**, in **Chapter 5** we investigated variation in gizzard mass and exploration behaviour. Contrasts between juveniles and adults provide support for the idea that environmental conditions play a key role in generating among-individual variation while the stabilization of traits in individuals occurred under constant conditions. In **Box 2** I reflect on what looks like 'confused' free ranging movements of a group of knots belonging to the *C. c. canutus* subspecies for which we curtailed their natural developmental trajectories. In **Chapter 6** we investigated if migratory movements are accompanied by a visual memory of previously visited places, for which preferences change seasonally. Although we found no evidence for such a mechanism, we did discover that knots are able to distinguish and memorize projected landscape images.

In **Chapter 7** I discuss how we could combine high definition tracking of individual birds with insights obtained from standardized assays to stimulate research into the cognitive aspects of migratory behaviour.

Samenvatting

Het doel van dit proefschrift (titel: Why *K*not? Exploration of Variation in Long-Distance Migrantion) is om de kennis te vergroten over hoe individuele kanoeten hun persoonlijke migratiestrategie ontwikkelen. In deze samenvatting wil ik de kern van dit onderzoek kort toelichten. Ik geef eerst kort een beschrijving van de leefwijze en verspreidingsgebied van de kanoet aan de hand van eerder gepubliceerd onderzoek. Vervolgens ga ik dieper in op mijn eigen onderzoek. Ik beantwoord verschillende type vragen over het trekgedrag van kanoeten. In het eerste gedeelte van dit proefschrift breng ik de trekroutes van twee ondersoorten van de kanoet in kaart (**Hoofdstuk 2, 3** en **Box 1**). In het tweede gedeelte (**Hoofdstuk 4-6** en **Box 2**) bestudeer ik vervolgens hoe variatie in fysieke eigenschappen (vlieg- en maagspier grootte) en gedrag (exploratie-, migratiegedrag en habitat voorkeur) tussen verschillende kanoeten zich ontwikkelen. In de laatste paragraaf van deze samenvatting geeft ik beknopt de conclusies van mijn proefschrift weer

Wat is een Kanoet?

Dit proefschrift gaat over Kanoetstrandlopers (*Calidris canutus*), in het Nederlands meestal kortweg *kanoet* genoemd (zie Hoofdstuk 1; Figuur 2 voor een afbeelding). Kanoeten wegen gemiddeld 120 gram en zijn ongeveer zo groot als een merel. Kanoeten bevinden zich het grootste gedeelte van het jaar in intergetijdengebieden zoals de Nederlandse Waddenzee. Toch hebben de meeste Nederlanders nog nooit een kanoet gezien, omdat de vogel kilometers uit de kust gebruik maakt van de droogvallende wadplaten. Tijdens laagwater doorzoeken ze op die wadplaten het wad, op zoek naar nonnetjes en kokkels die ze met schelp en al doorslikken. Vervolgens gebruiken de vogels hun sterke spiermaag om de schelpen te kraken en het vlees te verteren. De schelpresten poepen ze gewoon weer uit.

Wat is het verspreidingsgebied van de kanoet?

Kanoeten leven ook in andere intergetijdengebieden in de wereld, zoals in Zuid-Amerika, Australië, China en West-Afrika. De kanoeten in die landen zien er op het oog niet anders uit dan de kanoeten in de Nederlandse Waddenzee, maar vormen toch verschillende groepen, ook wel ondersoorten genoemd. Dat is het gevolg van de geografische verspreiding die de kanoeten aanhouden. Er zijn op dit moment zes verschillende kanoetenondersoorten bekend waartussen nauwelijks uitwisseling plaatsvindt (zie Hoofdstuk 1; Figuur 3) . Wat alle kanoeten gemeen hebben, is dat ze het grootste deel van het jaar in relatief warme intergetijdengebieden verblijven om vervolgens in het voorjaar duizenden kilometers naar het noorden te vliegen om te broeden op de Arctische toendra.

In de Nederlandse Waddenzee komen twee ondersoorten van de kanoet voor: de 'Russische Kanoet' (*C. c. canutus*) en de 'Canadese kanoet' (*C. c. islandica*). De Russische kanoet overwintert langs de west Afrikaanse kust, om precies te zijn in het Parc National du Banc d'Arguin in Mauritanië. Tijdens de voorjaarstrek eind mei, begin juni vliegen deze kanoeten in een keer van West-Afrika naar de Nederlandse Waddenzee om uit te rusten en bij te eten. Na drie weken vliegen de kanoeten verder noordwaarts naar hun broedgebieden op de Russische toendra. De Canadese kanoet verblijft de hele winter in Noordwest Europa, vooral in de Nederlandse Waddenzee en op de wadplaten langs de Engelse kust. In het voorjaar vliegen deze kanoeten via IJsland naar de broedgebieden in Noord-Canada, vlak bij de Noordpool (Zie Hoofdstuk 2; Figuur 1). Na het broedseizoen, halverwege augustus, begint de reis opnieuw, maar nu in omgekeerde volgorde.

Hoe kan een kanoet zo ver vliegen?

Om de duizenden kilometers lange vliegreizen succesvol te volbrengen ondergaan kanoeten elk jaar grote fysieke veranderingen. In het voorjaar moeten kanoeten heel veel eten om brandstof op te slaan,

ook wel *opvetten* genoemd. Door het opvetten worden ze bijna twee keer zo dik als in de winter en kunnen ze duizenden kilometers vliegen zonder tussendoor te stoppen. Daarnaast ruilen de vogels hun witgrijze verenkleed in voor een felrood verenkleed met zwarte stipjes. Met een kleurrijk verenpak kunnen de kanoeten een partner verleiden en zich beter verstoppen op de toendra. Omdat de zomer in de Arctisch kort is, komt de timing van de fysieke aanpassingen erg nauw. Immers, wanneer een kanoet te laat aankomt in het broedgebied zal deze niet genoeg tijd overhouden om kuikens groot te brengen voor het weer winter wordt.

Naast fysieke aanpassingen spelen ook de omstandigheden onderweg een belangrijke rol bij de vraag of een kanoet die route kan vliegen die hij van plan is te vliegen. De beschikbaarheid van geschikte rustplaatsen langs deze route, bijvoorbeeld, of onvoorspelbare veranderingen in het weer bepalen of een kanoet veilig én op tijd aankomt in het broedgebied. Dit alles maakt het trekgedrag complex en kwetsbaar voor veranderingen in de omgeving. Doordat kanoeten (en alle andere migrerende vogels) rekening moeten houden met deze onvoorspelbare jaarlijkse omgevingsvariatie, ontdekten biologen tot hun verrassing dat migrerende vogels met ervaring over het algemeen heel consequent zijn in bijvoorbeeld hun timing of het gebruik van rustplaatsen tijdens migratie.

Dit Proefschrift

Welke trekroutes volgde kanoet Paula?
In **Hoofdstuk 2** en **3** presenteer ik samen met collega-onderzoekers voor het eerst de exacte trekroutes van vrijlevende kanoeten van de *C. c. islandica* en *C. c. piersmai* ondersoort die wij met behulp van hele kleine satellietzenders in beeld gebracht hebben. Deze zendertjes, die maar 2 gram wegen en door middel van een klein zonnepaneel van stroom worden voorzien, worden bevestigd op de rug van een kanoet

met behulp van een minutieus afgesteld rugzakje van een duurzaam materiaal (zie **Box 1** voor de details). In **Hoofdstuk 2** volgen we kanoet Paula, die tot de eerder genoemde *C. c. islandica* ondersoort behoort. Gedurende twee opeenvolgende broedseizoenen vloog Paula vier keer over de ijskap van Groenland met een zendertje op haar rug. Door de afgelegde route in detail te bekijken, komen we tot een niet eerder ontdekte conclusie. Paula vindt niet alleen haar weg met behulp van een intern kompas waarmee ze op haar voorgenomen route kan blijven, maar Paula kan ook haar vliegrichting aanpassen wanneer de omstandigheden daarom vragen. Bijvoorbeeld wanneer zij in een gebied met sterke tegenwind terecht komt.

Wat gebeurt er in de Gele Zee?

In **Hoofdstuk 3** volgen we verschillende kanoeten van de *C. c. piersmai* ondersoort. Kanoeten van deze ondersoort verblijven het grootste gedeelte van het jaar op de intergetijdegebieden langs de Noordwest kust van Australië. Wanneer ze aan de noordwaartse migratie beginnen, vliegen ze onder andere via de Gele Zee in China naar hun broedgebied op de Nieuw-Siberische Eilanden in Rusland. In tegenstelling tot de vijf andere ondersoorten, stoppen vogels van deze kanoetenondersoort regelmatig op weg naar het broedgebied. Deze pauzes zijn daarentegen wel erg kort, immers, anders zou de reis te veel tijd in beslag nemen! Dit migratie patroon (zie Hoofdstuk 3, Figuur 1) , met meerdere 'stops' langs de Chinese kust, suggereert dat er in dat gebied veel geschikte plekken voor handen zijn om bij te eten.

Wat voor ontwikkelingen maakt een migrerende kanoet door?

We zien verschillen in trekroutes bij kanoeten met behulp van zenders, maar dat vertelt ons nog niet wat de oorzaken zijn van die variatie. Daarom heb ik in het tweede gedeelte van mijn proefschrift gestandaardiseerde metingen aan kanoeten in gevangenschap gebruikt om iets te kunnen zeggen over de oorzaken van variatie in trekgedrag

en fysieke eigenschappen.

In een speciaal voor steltlopers ontworpen faciliteit bij het Koninklijk Nederlands Instituut voor Onderzoek der Zee (NIOZ) hebben we gedurende een periode van twee jaar de gespierde maag van een groep kanoeten verkleind en vergroot door afwisselend voedsel van hoge dan wel lage kwaliteit aan te bieden. Met behulp van een echoscoop zagen we dat wanneer we de kanoeten zacht voedsel zonder schelp aanboden, ze dit gemakkelijk konden verteren en hun gespierde maag in grootte afnam. Wanneer we dezelfde vogels vervolgens een dieet gaven van harde, moeilijk verteerbare wadslakjes, nam de maaggrootte weer toe.

Waarom een grote maag meeslepen als je niet hoeft te eten?

De waarneming dat kanoeten hun maaggrootte kunnen aanpassen afhankelijk van het voedsel dat ze eten was al eerder gedaan. Echter, wat we nog niet eerder hadden waargenomen is dat de grootte van de vliegspier, die we ook konden opmeten met de echoscoop, ook reageert op de verandering in dieet. Terwijl kanoeten hun maag vergrootte om de moeilijk verteerbare wadslakjes te kunnen eten werd hun vliegspier kleiner. Andersom nam de vliegspier weer in grootte toe op de momenten dat de maaggrootte van de kanoeten af nam. Vermoedelijk ontstaat een dergelijke negatief verband tussen de maaggrootte en de vliegspier als resultaat van de migrerende levensstijl van de kanoet. Tijdens het migreren zijn kanoeten namelijk gebaat bij een grote, sterke vliegspier, maar aangezien ze in deze periode niet eten, hebben ze niet zoveel aan een grote, zware maag. Sterker nog, ze kunnen het best zo min mogelijk ballast mee nemen (**Hoofdstuk 4**)!

Hoe leren kanoeten voedsel te zoeken?

Kanoeten staan erom bekend dat ze van elkaar verschillen in maaggrootte, en dat er een verband bestaat tussen hun maaggrootte

en de manier waarop ze voedsel zoeken, ook wel exploratie gedrag genoemd. Om te onderzoeken welke factoren een rol spelen in de ontwikkeling van maaggrootte en exploratie gedrag bestudeerde we in **Hoofdstuk 5** dezelfde groep kanoeten als in **Hoofdstuk 4**. Deze gemengde groep individuen bestond uit onervaren-, jonge kanoeten die als juvenielen in de Nederlandse Waddenzee gevangen zijn en uit meer ervaren adulte kanoeten, tevens gevangen in de Waddenzee. Naast dat we van alle kanoeten in deze studie over een periode van twee jaar (waarin de jonge vogels volwassen werden) de veranderingen in maaggrootte in kaart brachten keken we ook naar de mogelijke veranderingen in exploratie gedrag in reactie op veranderingen in voedselkwaliteit.

We deden dit door het exploratie gedrag van individuele kanoeten te bekijken in een grote kooi waarin we het natuurlijke wad zo goed mogelijk nabootsten. We onderzochten vervolgens of individuen met verschillende maaggrootte ook verschilden in exploratie. Door vervolgens de uitkomsten van de groep adulten en juvenielen met elkaar te vergelijken, konden we bijhouden hoe de individuen van elkaar gaan verschillen, maar ook of deze verschillen het gevolg zijn van leeftijd, ervaring, of dat de verschillen ontstaan als gevolg van de kunstmatige omstandigheden in gevangenschap. Zo ontdekte we dat de groep juveniele vogels die opgroeide in een minder gevarieerde omgeving, ook minder variatie in exploratie gedrag liet zien dan de groep adulten. Maar ook dat hoewel het in eerste instantie misschien makkelijker lijkt om veranderingen in gedrag teweeg te brengen, het tegenover gestelde gebeurde. De kanoeten bleven consequent in hun exploratie gedrag, en dat terwijl hun maaggrootte zich wel aanpaste aan de (voorspelbare-) omstandigheden in gevangenschap.

Kunnen kanoeten migreren leren?

In **Box 2** laat ik zien wat de rol is van ervaring op de ontwikkeling van natuurlijke migratieroutines. Ik beschrijf dat juvenielen van de *C.*

c. canutus ondersoort, die opgegroeid zijn in gevangenschap nabij de Nederlandse Waddenzee in plaats van aan de West Afrikaanse kust, verward lijken over hun natuurlijke migratie gedrag wanneer zij, inmiddels volwassen, weer worden vrijgelaten in de Waddenzee. Terwijl hun soortgenoten al lang zijn doorgetrokken naar de West Afrikaanse kust blijven sommige van 'onze' vogels de hele winter in de Waddenzee. En ook op lokaal niveau, binnen de Nederlandse Waddenzee, worden ze regelmatig terug gezien op 'vreemde' plekken waar wilde kanoeten zelden worden gezien, zoals op het Noordzeestrand van Texel. Het lijkt er dus op dat de omgeving waarin jonge kanoeten opgroeien belangrijk is voor de ontwikkeling van het trekgedrag op latere leeftijd.

Wat motiveert kanoeten om te migreren?

In **Hoofdstuk 6** onderzoeken mijn collega's en ik of kanoeten naast grote veranderingen in fysieke gesteldheid ook mentale veranderingen ondergaan wanneer zij zich voorbereiden op migratie. We vroegen ons af of kanoeten in het voorjaar gemotiveerd raken om naar de toendra te vliegen, een beetje zoals wij mensen aan het eind van een lange reis reikhalzend kunnen uitkijken om weer naar huis te gaan. Een dergelijk verlangen naar een verre bestemming zou migrerende vogels kunnen motiveren om twee maal per jaar over enorme afstanden te migreren. Door de ene groep kanoeten aan te leren dat ze voedsel kunnen vinden naast de projectie van een foto van het wad, en een andere groep naast een foto van het bos hebben we kunnen aantonen dat kanoeten in staat zijn om projecties van verschillende landschapstypes te onderscheiden. In het vervolg experiment, waarin de kanoeten niet beloond werden met voedsel, hebben we geen bewijs gevonden voor een seizoensgebonden voorkeur voor afbeeldingen van het broedgebied dan wel van intergetijdengebieden.

Zijn er nog vragen?

In dit proefschrift heb ik migratie routines van kanoeten in kaart gebracht en gecombineerd met metingen aan individuen in een gecontroleerde omgeving om op verschillende manieren te laten zien dat de ontwikkeling van individuele migratie strategieën beïnvloed worden door de omgeving.

Wat ik hierdoor heb kunnen laten zien is dat kanoeten niet alleen in hun huidige gedrag beïnvloed worden door hun omgeving maar dat ook de reeds opgedane ervaring van belang is voor toekomstige beslissingen; zónder dat dit gepaard gaat met een verlies in aanpassing vermogen! In **Hoofdstuk 5** noem ik dit 'kanalisatie' van gedrag; een ontwikkeling van gedrag die vergeleken kan worden met een leerproces. De cognitieve aspecten van migratie worden momenteel sterk onderbelicht. Ingegeven door de bevindingen in dit proefschrift pleit ik voor een meer holistische benadering van wetenschappelijk onderzoek naar migratie gedrag waarin ook de cognitieve aspecten van migratie een rol krijgen.

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De eerste keer dat ik een kanoet zag was op 15 januari 2012 op een wadplaat genaamd Ebelgh Khaznaya in natuurreservaat Banc d'Arguin in Mauritanië. Ik was daar in het kader van mijn Master project op het NIOZ, toen nog als student biologie aan de Universiteit van Amsterdam. Samen met een handjevol wetenschappers van het NIOZ was ik er de dag ervoor vanaf Schiphol naar toe gereisd. Terwijl ik die dag naast Emma op mijn snowrackets voor het eerst van mijn leven door een telescoop naar foeragerende kanoeten stond te kijken, had ik nooit kunnen bedenken dat deze vogels, maar vooral ook deze mensen en de grotere groep warme, gepassioneerde, hard werkende mensen die ze representeerden, zo'n groot onderdeel van mijn leven zouden uitmaken in de jaren die volgden. Hoewel mijn naam voorin dit proefschrift staat, is dit boekje in feite het resultaat van vele kleine stapjes die ik met de hulp van nog veel meer mensen heb kunnen maken. Ik zeg dan ook uit de grond van mijn hart dat ik van elke stap heb genoten.

Theunis, ook jij en ik ontmoetten elkaar voor het eerst in 2012 in Iwik. Ik was meteen onder de indruk van jou (emotionele) betrokkenheid bij de vogels en hun habitat. Gebogen over een kanoet met pootkramp vertelde je me over een idee voor een risicovol experiment waar je al jaren mee rond liep en waarin je kanoeten plaatjes van landschappen wilde voorleggen. Dit soort cognitieve vraagstukken lagen helemaal in mijn straatje en zo kwam het dat ik na mijn eerste stage op het NIOZ bleef om het experiment dat aan de basis ligt van hoofdstuk 6, uit te voeren. Toen jij in 2014 de Spinoza prijs uitgereikt kreeg, greep jij deze mogelijkheid aan om mij een PhD aan te bieden om verder te gaan met dit onderzoek. Sindsdien hebben we veel verschillende projecten gedaan die samen de basis vormen voor dit proefschrift. Dankjewel voor je rotsvaste vertrouwen in mij en mijn 'vogeltjes-handen'. Doordat jij ondanks je overvolle agenda altijd tijd vrij maakte voor een motiverende speech of om mijn manuscripten van commentaar te

voorzien heb ik de afgelopen jaren veel meer voor elkaar gekregen dan ik zelf voor mogelijk had gehouden.

Dear **Kim**, your return at NIOZ and our ensuing collaboration was as unexpected as it was fruitfull. After a footloose first PhD year I really enjoyed being part of a team again. Thank you for making me feel that I was not just your pupil but also a valuable partner in crime. Also, after your move to Edmonton and the University of Alberta you have always been very supportive and I am very grateful to have you as my co-promoter. I admire your (work-) ethics and dedication, even if I only got a little of that I consider myself a lucky scientist.

Dear professors of the reading committee (**Judy Shamoun-Baranes, Bart Kempenaers, Barbara Helm**) thank you for taking the time to assess my thesis. **Judy**, I am a bit sad that this is probably my last thesis for you to assess.

Emma, in een eerste versie van dit dankwoord kwam jouw naam in elke paragraaf voor. Logisch, je was namelijk overal bij! We zaten samen vast in de modder van de Banc d'arguin, Vistula Mouth, de Waddenzee en het Estlandse hoogveen maar we delen ook al jaren lang lief en leed op kantoor. Ik bewonder je kennis en passie voor het wad die je duidelijk met de paplepel is ingegoten. Dankjewel voor je vriendschap en steun als ideale paranimf tijdens mijn gehele promotietraject.

Roeland, wat begon als een beetje grootspraak en een oefenritje naar Workum eindigde in een epische fietstocht dwars over het Engelse continent. Iemand met wie ik dat kan, heb ik natuurlijk ook graag tijdens mijn promotie naast me staan. Jij bezit de gave om ogenschijnlijk moeiteloos zowel plezier als rust in de groep te brengen en ik ga dan ook graag bij je te raad. Dankjewel.

In de twee stage jaren voorafgaand aan mijn PhD project woonde ik op campus de Potvis. Een tijd waaraan ik naast dierbare herinneringen ook goeie vrienden over gehouden heb. Niet geheel toevallig hebben

al deze Potvissers ondertussen ook (bijna) de doctors titel op zak. **Nol**, het (buiten-)volleybal en praatjes door de jaren vormen een rode draad door mijn gehele NIOZ periode. **Estefania** ik zou nog steeds wel terug willen naar de Potvis tijd waarin we elke avond samen een kopje sleepytime-tea dronken, ik mijn balkon deelde met Bradley Cooper en we niet in het Nederlands konden communiceren behalve wanneer we spraken over 'lepelaar kots' en 'dooie kuikens'. **Ineke** jou enthousiasme voor de wetenschap en communicatie werkt aanstekelijk.

Lise, in 2014 hadden we allebei het geluk een zogenoemde 'Pay-Back PhD Positie' te bekleden. Ik (inmiddels de Potvis ontgroeid) nam dankbaar gebruik van de mogelijkheid om doordeweekse de logeerkamer in je nieuwe huis te betrekken. Toen Steven en ik een jaar later zelf een huisje kochten in Alkmaar en ik niet langer op het eiland hoefde te overnachten, werd mijn plek al snel ingevuld door **Loran**. Ik ben jullie beide erg dankbaar dat er sindsdien altijd een plekje voor mij is gebleven op de Molenstraat. Hoewel we elkaar soms lange tijd niet 'zagen' voelde de volleybal avonden en de gesprekken aan de eettafel altijd aan alsof het nooit anders is geweest. Lise, het doet me veel plezier dat we onze proefschriften in dezelfde periode afgerond hebben. Ik kijk er naar uit om op 30 oktober naast je te staan tijdens jouw verdediging! **Suus**, dank je wel voor alle etentjes en late-night logeer partijen. Ik ben je ook dankbaar dat ik eerder dit jaar jou paranimf mocht zijn. Mocht mijn verdediging door de corona pandemie toch online plaats vinden heb ik dankzij jou toch een beetje gevoeld hoe het is om op het podium voor een groot publiek te staan.

Toen ik naar Alkmaar verhuisde en daardoor dagelijks heen en weer moest varen met de veerboot belandde ik als vanzelf in een nieuwe NIOZ dimensie. Zo begon de 'werk-' dag soms al op de N9, of op het TESO parkeerterrein waar ik stiekem wedstrijdjes reed met niets vermoedende collega's. Later, toen ik mijn VW Lupo inruilde voor de trein, marcheerde we in een steeds verder uitdijende Alkmaar

en vriendschap waren de begindagen van mijn PhD project een stuk saaier en eenzamer geweest. Dankjewel. **Anne**, jou kan ik niet genoeg bedanken. Jouw kennis en kunde over de kanoet en het wad zijn eindeloos, al geef je ze niet graag prijs. Op de spannendste momenten vertrouwde ik op jou. Want als jij zegt dat ik moet springen, dan spring ik. **Anita**, dankjewel voor de goeie gesprekken, de ondeugende grappen en de schaatsoefeningen op het ijs én in de NIOZ gang! **Kees**, bedankt voor je aanwezigheid en de heerlijke dagen in de kolonie. Ik ken niemand die zo erg zijn eigen koers volgt en tegelijkertijd zo goed is in mensen verbinden. Our lonely people dinners were momentous. **Petra**, dankjewel voor je betrokkenheid bij mijn project en het steuntje in de rug wanneer dat nodig was. Onze jacht op het noorderlicht met Jelle en Theunis zal ik niet snel meer vergeten. Het was me een genoegen om in 2018 samen met jou, **Bob** en vele andere de IWSG in Workum te organiseren.

Lieve **Piet**, jij bent denk ik de enige persoon die ik ken die met net zoveel (of misschien wel meer) enthousiasme als ikzelf kan vertellen over die ene 'dood-normale-vogel' die je onderweg tegen kwam en die toen iets 'bijzonders' deed (en daarna gewoon weer weg vloog). Van jou heb ik geleerd dat deze waarnemingen net zo belangrijk kunnen, én mogen, zijn als doordachte wetenschappelijke experimenten. Dankjewel voor je vriendschap. **Allert**, toen ik aan mijn promotie traject begon was jij de jouwe net aan het afronden. Since then you've built a movement-lab together with **Selin** and **Pratik** that seems to be growing by the week. Thank you for 'having' the *canutus* juveniles in the WATLAS system during the last year of my project. Consequently, their first free ranging movements could be part of this thesis after all (Box 2). Onze openhartige gesprekken over de wetenschappelijke wereld zijn me heel dierbaar, ook, of misschien wel juist, de iet wat wazige varianten in de Potvis, Sporthal, Slock, Luca en de NVG. **Roos**, dankjewel dat je aan de bel trekt wanneer dat nodig is. Het bezoek van Emma, Suus en mijzelf tijdens je Postdoc in Oxford en onze

gemeenschappelijke roadtrip naar het congres van de BOU (Britisch Ornithological Union) blijft een memorabel onderdeel van mijn PhD. **Tamar** dankjewel dat je me mee nam om lepelaars te ringen. Het zijn dit soort ervaringen waardoor ik zo enorm geniet van ons werk. Ik heb ooit eens gelezen dat ik mijn Pay-Back positie te danken heb aan jouw en **Matthijs**' verdediging! **Jan**, tijdens mijn eerste stage in de Banc d'Arguin zetten ik (aan jouw hand) mijn eerste stappen in de wereld van de kanoet. Met je grenzeloze enthousiasme en werkelijk onuitputtelijke energie maak je het onmogelijke, mogelijk. Toen ik mij in mijn PhD meer richtte op ontwikkeling en cognitie heb je mij de ruimte gegeven om dit te doen. Tegelijkertijd heb je mij geïntroduceerd bij Włodek en Agnieszka en zo de expedities naar de Vistula Mouth in Polen gefaciliteerd. Ik ben je voor beide ontzettend dankbaar en ik kijk uit naar de mooie kanoeten verhalen die we in de toekomst hopelijk nog gaan schrijven. Dear **Martin**, I feel privileged to have been able to work with you during your Marie Curie Postdoc at NIOZ. Thank you for shaking up rusty procedures and inefficient habits as well as many other joyful moments.

Fortunately I was able to work with a great bunch of (Master) students and volunteers over the years (**Joey, Darren, Petra, Robert, Tim, Baptiste, Luc, Demy** and **Jessica**). Thank you for all your hard work and dedication. Joey and Darren, when Kim and I set-up our long-term experiments with a big group of knots in captivity (Chapter 4 and 5) we couldn't have wished for a better team to do this with. In the second year of the experiments, Petra, Demy, Luc and Baptiste formed a close and productive team that continued the exploration work while collecting hours and hours of behavioural data on the side that still require writing up. **Luc**, ik ben nog steeds heel blij dat jij kon blijven als dierverzorger. Ondertussen ben je niet meer weg te denken uit de afdeling. Dankjewel voor je grote bijdrage aan dit proefschrift. Jouw harde werk heeft het mij op veel momenten, bijvoorbeeld bij de backpack tests (Box 1), veel gemakkelijker gemaakt. **Jan W.**., tige

tank voor je hulp met de kanoeten, de experimenten en de mooie foto's. Steven en ik denken vaak met een warm gevoel terug aan onze vakantie bij jullie in Edmonton en natuurlijk vooral aan ons tripje naar Banff. **Job**, dankjewel dat je me regelmatig hebt meegenomen tijdens vangacties in de Waddenzee. Terwijl ik over het wad liep voelde ik me altijd de koning te rijk. Jouw inzet voor het ring onderzoek is ongeëvenaard. **Benjamin**, your extraordinary number of red knot color ring resightings (almost 30 000 in 3 years?) deserve special acknowledgements here. On one occasion you discovered red knot Paula on the mudflats around Griend only a few hours after her return from Ellesmere Island, beating the high-tech satellite transmitter on her back in both accuracy and speed. **Thomas**, al ruim 12 jaar kruisen onze paden elkaar. Net zo lang bewonder ik je kwaliteit om vaagheid te omarmen maar dat tegelijk verdomd duidelijk te verwoorden. Dankjewel dat je altijd zin hebt om mee te denken, dat je me mee sleepte naar een lezing van Marianne Thieme en in sneltreinvaart St. Andrews hebt laten zien. Ik ga ervan uit dat onze wegen zich ook de komende jaren zullen blijven kruisen. **Ginny**, thank you for sharing your profound knowledge on backpack techniques, tracking and the Australasian flyway with me. I'm looking forward to complete Chapter 3 together for publication. **Julia** and **Eldar**, thank you for readily sharing your extensive knowledge on statistics and migratory systems but also for our worldly conversations during shared dinners. Losing you to Groningen is a great sacrifice for our group but I'm happy to see that many collaborative projects and friendships continue to exist.

Lieve **Jan-Berend**, vooral tijdens de tweede helft van mijn PhD, toen ik niet meer het veld of het lab in hoefde, was jouw steun van essentieel belang. Dankjewel voor alle potjes volleybal, baantjes in het zwembad, stroopwafels, relativerende suggesties en onuitputtelijke aanmoedigingen om het geheel gewoon even op te schrijven. **Meta**, dankjewel voor het meedenken. Vaak had je mogelijke problemen met bijvoorbeeld de NIOZ auto's of mijn toegangspasje al ondervangen

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Hoewel mijn project ondergebracht was bij het NIOZ was mijn promotie traject officieel geborgd bij de Universiteit van Groningen. Mijn dank gaat dan ook uit naar de medewerkers van de RUG (**Corine, Joyce, Betsy, Ingrid**) die, ondanks het feit dat ze mij maar zelden zagen, zorg hebben gedragen voor de registratie van mijn project en verdediging via Hora Finita. Dank ook aan **Yvonne**, **Jesse** en **Jeroen O**. die voor een belangrijke verbinding zorgen tussen Texel en Groningen.

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Since my first attendance in 2012 (Wilhelmshaven) I did not miss a single conference organized by the International Wader Study Group. Among the attendees many inspiring wader professionals (**Triin, Camilo, José, Veronica, Afonso, Sölvi, Jorge, Michał, Linus, Johannes, Simeon, Martin, Josh, Ana, Mohammed, Wim & Jos and many others**) that make every edition feel like a reunion weekend with friends.

Early 2018 I participated in the Wader and Tern expedition in North-West Australia to catch and track the red knots that are featured in

Chapter 3. I would like to thank **Chris and Clive's team** as well as the other participants of the expedition for facilitating this great new experience and getting to know this amazing country. Special acknowledgments are in place for **Chris** and **Kerry** for their hospitality in the field and at their home. The expedition wouldn't have been the same without **Drew**, thank you for all your help and good company. When cyclone Kelvin forced us to drop the work and lay low for a while **Marcel** took me to the pool to teach me the basic principles of the freestyle stroke. A simple gesture with life changing consequences because it facilitated me to finally pursue my long-aspired triathlon ambitions.

Lee, although we've never met in person we exchanged emails almost on a daily basis throughout most of my PhD project. Thank you for a fruitful and pleasant collaboration. **Jerry**, thank you for sharing your exceptional take on the world and science in particular. Your influence did not remain restricted to Chapter 6 but is tangible throughout my thesis. Thank you for proofreading an earlier version of Chapter 1. Dear **Agnieszka, Włodek** and ringing group **Kuling**, thank you for your hospitality at your field station at the Vistula Mouth and Gdansk University. Fresh mealworms, genuine seawater or guarded access the university in the middle of the night, you've made it all possible.

Tijdens mijn PhD-project ben ik meerdere keren uit de brand geholpen door ringgroep **Calidris** van Schiermonnikoog. Ik denk met een glimlach terug aan hoe ik eens met mijn verhuisdozen met de eerste boot op Schier aankwam terwijl jullie (**Kees, Koos, Symen, Thomas, Irene**) me met kleine oogjes op me stonden te wachten om de vangst van die nacht over te dragen. Waarvoor hartelijk dank! Ik ben mijn mede bestuursleden van de **NOU** (Nederlandse Ornithologische Unie) dankbaar voor de introductie in de wereld van de Nederlandse vogelaars. Mijn taak om tijdens het de jaarlijkse SOVON dag een NOU symposium te organiseren heb ik met veel plezier met **Thomas**

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