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# Ecoimmunology perspective of host-parasite interactions in *Limosa limosa* across its migratory flyway

Tese de Doutoramento em Biociências, especialização em Ecologia,
orientada pelo Professor Doutor Jaime Albino Ramos e pela Professora Doutora Helena Westerdahl,
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# Ecoimmunology perspective of host-parasite interactions in *Limosa limosa* across its migratory flyway

UNIVERSITY OF COIMBRA

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# Preface: Birds flight

Since I was a young girl I have had a strong fascination by birds and their life style. Birds are beautiful animals with compelling colours, ornaments and behaviours. However, my curiosity was mostly focused on bird flights and how they undertake migratory movements, behaviours which scale and regularity has no other analogy in the animal world. Their ability to fly, scales down our own perspectives of distance, and gives birds the possibility to move between widely separated areas at different seasons while returning to the same places year after year. I suppose that my child fascination and the way I discovered the world by that time, have matured and that this thesis was then inspired by my outgrowing fascination and "never ending questions" about the drivers of migration and bird ecology.

Bird migration has for long puzzled researchers and posed many questions. How do birds withstand such long non-stop flights? How do they navigate? How do they prepare their bodies for these journeys? In recent years, a new discipline in the study of migration addresses how do migrant birds deal with such different pathogen communities found along the migratory flyways, and how do they cope with infection considering how energetically demanding and busy their life style is. Before I go into further details, I should define the type of migrations and birds I focused my work on. In this thesis, the studied migration type is the one that involves long journeys, between non-breeding grounds at lower latitudes and breeding grounds at higher latitudes, in which the paths/directions birds use along the way are somehow fixed or encoded in their genes. Seasonal changes are the main drivers, and therefore these migrations are behavioural adaptations to exploit seasonal peaks of resource abundance and avoidance of their depletion when climate conditions are less favourable (Newton 2008).

Waders (or shorebirds), gulls and auks include some of the fastest and agile fliers. As all belong to the Charadriiformes order, some of the commonest birds included are the ones we see dwelling in coastlines and freshwater sites, though some species can also be found inland (Delany et al. 2009). Waders make up nearly two thirds of the total number of species in the order Charadriiformes, they typically have long legs and beaks, feed by probing in the

sand or mudflats and include some long-distance marathon migrant species (*e.g.* the Bar-tailed godwit, *Limosa lapponica* and Artic tern, *Sterna paradisaea*). What makes them particular interesting for this study is the wide range of migratory strategies that result in striking differences on pathogen exposure, which is relevant from a host-pathogen perspective (Delany et al. 2009; Piersma 1997; Piersma 2003; Clark et al. 2015).

# Summary

For long-distance migrant birds, ecological changes along the flyway and strenuous work, can unbalance immune investments and thereby increasing the vulnerability to disease and reducing survival. Despite an essential self-maintenance component, immunity comes with costs as well as benefits. At an ecological context (*i.e.* limited energy), trade-offs are generated between immunity and other competing physiological components, leading to variations on immune response across time (annual-cycle) and space (different environments). Moreover, immune responses also vary between species and are optimized according to specific situations (*e.g.* breeding) of an individual´s life, to maximize survival and fitness.

Many ecoimmunology studies are focused on understanding the general patterns of immune responses in free-living birds, and what mechanisms mediate the changes in disease susceptibility, which in turn may affect population dynamics and survival. Information regarding these immunologic trade-offs remain largely unknown, and thus becoming of paramount importance when contextualized with climate change effects over migration ecology and the distribution of animal diseases.

The work comprised in this thesis approaches three main subjects: migratory strategies, investments in immune responses and molecular evolution, to address the general question of how differences in environmental pathogen pressure shape the innate and adaptive immunity of a free-living bird species. The long-distance migrant Black-tailed godwit (*Limosa limosa*; hereafter godwit), was used as a study model, since the two subspecies, nominate (*Limosa limosa limosa*) and Icelandic (*L. l. islandica*), vary on migratory strategy and habitat-use, which consequently differ in pathogen pressure. Here, I summarize the main contributions of my work for the ecoimmunology research area.

On chapter I, I tested whether the strength of immune response is indeed correlated with the environmental pathogen risk that varies accordingly to the migratory strategies and habitat use of the Black-tailed godwit. The work focused on the innate baseline immunity parameters such as leukocyte counts, haptoglobin levels and complement activity and

natural antibodies. I showed that innate baseline immunity changes during the annual cycle of godwits in response to seasonal demands, availability of resources and physiological trade-offs. The results suggest that investment in immune defence and the strategies deployed, vary also in response to the ecological context and the risk of getting infected. These trade-offs between immune function and other physiological components become more apparent during energetically demanding periods, such as during the breeding season in which the Icelandic godwits afford a downregulation of innate responses by occupying parasite-poor breeding areas. During winter, nominate godwits increased some immune parameters thought to be important for controlling the fast replicating pathogens of tropical areas. Habitat differences under the same latitudinal scale also influenced cellular responses, with nominate godwits having higher levels of phagocytic cells than the Icelandic godwits. Our results indicate that these immune adjustments and strategies are not transversal to all bird species, but instead rather unique. Nonetheless, one trend seems to affect several species in the same way. As expected, migration led to an overall immunosuppression of the innate immune response, which may underline a higher vulnerability to disease during these periods.

On chapter II, the MHC-I gene of the Icelandic godwit is characterized based on Sanger and ultradeep Illumina MiSeq sequencing. In part such characterization was done on the α2 domain (exon 3) of this gene, which is known to be highly polymorphic and coding part of the glycoproteins that recognize pathogens. I found 47 new alleles of MHC-I exon 3, and all of a putatively classical nature (functional). Icelandic godwits have between one and four loci, with at least three being expressed, and the gene organization of classical loci is quite similar to their closest relative, the Red knots (*Calidris canutus*). Comparing to other Charadriiformes species, the alleles have a lower polymorphism and few sites subjected to positive selection, which I suspect to be a reflection of a lower pathogenic pressure that individuals experience along their migratory flyway.

On chapter III, the role of pathogens as drivers of the diversifying selection of MHC genes is analysed. By using ultradeep Illumina MiSeq sequencing we compared two godwit subspecies in terms of MHC-I exon 3 diversity and polymorphism. Both nominate and Icelandic godwits overlapped in terms of number of alleles (and loci) per individual, but

nominate birds had significantly higher number of alleles and polymorphism. However, a population demography effect could partly explain the differences seen at the MHC-I diversity levels between subspecies, because the neutral gene markers of Icelandic godwits are far less diverse. Nonetheless, the number of positively selected sites for the nominate godwit was twice as high as those found for the Icelandic godwits, a pattern unlikely to be confounded by different effective population sizes. Differences seen on positive selection, suggest a stronger balancing selection for the nominate godwit, which is an evidence of their adaptation to pathogen-rich habitats.

Chapter IV is focus on sexual signals and evaluates whether they truly advertise the quality of mates and their capacity to fight-off pathogens. I tested whether investment on innate immunity could be reflected by the melanin-based sexual characters of godwits. On our study system these trade-offs become especially relevant because godwits overlap moult and spring migration, and under such energetic constraints investment into a colourful breeding plumage may not be favoured. Results indicate that some plumage features of male and female godwits were indeed linked to soluble parameters of innate immunity and were not cost-free. Moreover, the signal was honest for males but not for females, a difference that may be related to sex-specific energetic demands and roles undertaken in breeding. Our results suggest that females select males that are better at combining moult and migration and more able to fight infections.

Chapter V tackles a more pratical issue faced by researchers that work with free-living birds, and with no immediate access to laboratory facilities. Immunological assays have become a widespread tool for an integrate approach of immune variation, but it was unknown whether post-sampling freeze-thawing cycles could affect the final outcome of the assays. Repeated freeze-thawing cycles can be a common practice when testing the same individual samples in multiple assays, but no information was available for the deterioration effects on bird's blood caused by these cycles, when they were known to cause deterioration on some human blood components. An experimental approach testing post-sampling handling and methodological issues was implemented, and overall results showed that plasma (and serum) samples remain stable after repeated freeze-thawing cycles, and thus the indices of immune function are mostly unaffected. We also showed that methodological

deviations of the protocols of these assays, caused no substantial variations on the final results. Nonetheless we advise researchers to follow best laboratory practices and standardization, to avoid introducing artificial bias on their assays.

**Keywords**: Ecoimmunology, long-distance migration, waders, pathogen pressure, innate immunity, adaptive immunity, molecular evolution

# Resumo

Para aves migradoras de longa distância, as mudanças ecológicas ao longo da rota migratória, aliadas ao esforço físico, podem desequilibrar os investimentos em imunidade, levando a uma maior a vulnerabilidade à doença e reduzindo a sua capacidade de sobrevivência. Apesar de a imunidade ser um componente fisiológico essencial de auto-preservação, esta implica custos, bem como benefícios. Num contexto ecológico e com energia limitada, são gerados *trade-offs* entre a imunidade e outros componentes fisiológicos, dando origem a variações da resposta imune ao longo do tempo (ciclo anual) e do espaço (diferentes ambientes). Para além disso, as respostas imunitárias também variam de acordo com a espécie e são optimizadas em função de situações específicas da vida de um indivíduo (por exemplo, reprodução) para maximizar a sobrevivência e o *fitness*.

Muitos estudos em ecoimunologia focam-se na compreensão dos padrões globais de respostas imunes em aves selvagens e nos mecanismos que medeiam as mudanças na susceptibilidade à doença, o que por sua vez poderá afectar a sua sobrevivência e a dinâmica populacional. Infelizmente a informação relativa a estes *trade-offs* imunológicos permanece em grande medida desconhecida, tratando-se portanto de conhecimento essencial quando contextualizado com os efeitos das alterações climáticas sobre a ecologia da migração e distribuição de doenças animais (zoonoses).

O trabalho englobado nesta tese foca três temas principais: estratégias migratórias, investimentos em imunidade e evolução molecular, para responder à pergunta de como é que as diferenças na pressão parasítica ambiental afectam a imunidade inata e adaptativa de aves selvagens. O migrador de longa distância Maçarico-de-bico-direito (*Limosa limosa*), foi usado como modelo de estudo, uma vez que duas das suas subespécies, nominal (*Limosa limosa limosa*) e islandesa (*L. l. Islandica*), variam na estratégia migratória e uso de habitat, que consequentemente difere na pressão parasítica. De seguida, resumo as principais contribuições do meu trabalho para a área da ecoimunologia.

No capítulo I, usando uma ave límicola como modelo, testei se a capacidade da resposta imune está de facto correlacionada com o risco de contrair doenças, uma vez que o risco difere com as estratégias de migração e habitat. O trabalho focou parâmetros de imunidade inata, tais como contagens de leucócitos, concentração de haptoglobina e actividade do complemento e dos anticorpos naturais. Demonstrei que a imunidade inata não é de natureza estanque, mas que varia ao longo do ciclo anual dos Maçaricos-de-bico-direito em resposta à estação do ano, disponibilidade de recursos e *trade-offs* fisiológicos. Os investimentos na imunidade e estratégias aplicadas também variaram em resposta ao risco de infecção. Os *trade-offs* entre a imunidade e outros componentes fisiológicos, tornam-se mais visíveis durante períodos energeticamente exigentes, como a reprodução. Nestes períodos, a subespécie islandesa de Maçarico-de-bico-direito suprimiu alguns componentes da imunidade inata, quando se encontrava em zonas onde o risco de infecção é baixo. Durante o inverno, a subespécie nominal de Maçarico-de-bico-direito, aumentou componentes da imunidade inata, que suspeitamos serem importantes para o controlo de agentes patogénicos de replicação rápida, típicos de zonas tropicais. Sob a mesma escala latitudinal, as diferenças de habitat também influenciaram as respostas celulares, onde a subespécie nominal de Maçarico-de-bico-direito apresentou níveis mais elevados de células fagocíticas, comparativamente aos Maçaricos islandeses. Os resultados deste trabalho também indicam que os ajustes e estratégias imunitárias não são transversais às várias espécies de aves, mas que pelo contrário são únicas de cada espécie. Há no entanto um padrão que parece afectar as variadas espécies da mesma maneira, mais concretamente durante a migração. Tal como esperado, a migração conduziu a uma imunosupressão geral da resposta inata, o que poderá indicar um período de maior vulnerabilidade à doença.

No capítulo II, o gene MHC-I da subespécie islandesa de Maçarico-de-bico-direito foi caracterizado com o auxílio da sequenciação de Sanger e Illumina MiSeq. A caracterização focou quase exclusivamente o domínio α2 (exão 3) deste gene, que é conhecido pelo seu polimorfismo e por codificar parte das glicoproteínas que reconhecem agentes patogénicos. Neste trabalho descobri 47 novos alelos do gene MHC-I, todos eles de natureza provavelmente clássica (ou funcional). Os Maçaricos islandeses têm entre um e quatro loci, com pelo menos três a ser expressos. A organização do gene MHC-I é bastante semelhante à

do seu parente mais próximo, a Seixoeira (*Calidris canutus*), mas comparado com outras espécies de Charadriiformes, os alelos tinham um polimorfismo menor e poucos locais sujeitos a selecção positiva. Este padrão parece ser um reflexo de os indivíduos experienciarem a uma menor pressão patogénica ao longo da sua rota de migração.

No capítulo III, aprofundámos o papel da pressão patogénica como origem da diversificação dos genes MHC. Com o auxílio da sequenciação Illumina MiSeq, comparámos as duas subespécies de Maçarico relativamente à diversidade e ao polimorfismo do exão 3 do gene MHC-I. Ambas as subespécies sobrepuseram-se no que toca ao número de alelos (e loci) por indivíduo, mas a subespécie nominal, tinha significativamente mais alelos e tendencialmente um maior polimorfismo. Diferentes tamanhos populacionais poderão explicar parcialmente as diferenças encontradas entre subespécies ao nível da diversidade genética, no entanto, o número de locais sujeitos a selecção positiva encontrados para a subespécie nominal, foi duas vezes superior ao encontrado para a subespécie islandesa. As diferenças no que toca à selecção positiva, sugerem que a selecção natural é mais forte para a subespécie nominal e portanto uma adaptação a habitats ricos em parasitas.

O capítulo IV centra-se nos sinais sexuais e avalia se eles realmente reflectem a qualidade dos parceiros e a sua capacidade em combater doenças. Para isso testei se o investimento na imunidade inata poderia ser reflectido pelos carácteres sexuais secundários da plumagem dos Maçaricos-de-bico-direito. No nosso sistema de estudo, estes *trade-offs* são particularmente relevantes porque esta espécie sobrepõe a muda da plumagem de reprodução com a migração de Primavera, e sob tais restrições energéticas, o investimento numa plumagem mais colorida pode não ser favorecido. Os resultados indicam que algumas características da plumagem dos machos e fêmeas, estão de facto, ligadas a componentes solúveis de imunidade inata e que estes investimentos na plumagem não estão livres de custos para o animal. Para além disso, o sinal foi honesto para os machos, mas não para as fêmeas, uma diferença que poderá estar relacionada com constrangimentos energéticos específicos para cada sexo, e pelos papéis desempenhados durante a reprodução. Por conseguinte, os nossos resultados sugerem que as fêmeas seleccionam, não só os machos que são melhores a combinar a muda da plumagem e a migração, mas também os mais competentes no combate às infecções.

O Capítulo V aborda uma questão mais prática enfrentada por investigadores que trabalham com aves selvagens e sem acesso fácil ou imediato ao laboratório. Os ensaios imunológicos tornaram-se uma ferramenta generalizada para uma abordagem integrada da imunidade, mas desconhecia-se até à data se os ciclos de congelamento-descongelamento pós-amostragem poderiam afectar o resultado final dos ensaios imunológicos. Ciclos de congelamento-descongelamento repetidos, podem ser uma prática comum para investigadores que pretendem usar as mesmas amostras em ensaios múltiplos, mas não havia qualquer informação disponível sobre se estes ciclos causariam deterioração no sangue das aves, embora já se soubesse que alteravam componentes do sangue humano. Neste trabalho foi implementada uma abordagem experimental para o tratamento pós-amostragem, bem como outras questões metodológicas, e os resultados indicaram que as amostras de plasma (ou soro) permanecem estáveis após ciclos repetidos de CD e que portanto, os componentes imunológicos permanecem inalterados. Mostrámos também que pequenas alterações metodológicas nos protocolos destes ensaios não causaram variações substanciais nos resultados finais. No entanto, aconselhamos os investigadores a padronizarem a análise e a seguirem as melhores práticas de laboratório, para evitar a introdução artificial de erros nos seus ensaios.

**Palavras-chave**: Ecoimmunologia, migradores de longa distância, limícolas, pressão parasítica, immunidade inata, imunidade adaptativa, evolução molecular

# General Introduction


**“Be as a bird perched on a frail branch**
**that she feels bending beneath her,**
**still she sings away all the same,**
**knowing she has wings.”**

**Victor Hugo**

# What is Ecoimmunology?

Despite still in its infancy, over the past 15 years the amount of research conducted in ecoimmunology has increased massively. The role of parasites in the evolution of life-history traits along with susceptibility to a variety of infectious diseases, has become of increasing interest to both ecologists and evolutionary biologists. At the same time, the immune system turned to be an important research area for understanding changes in disease susceptibility[1], what mechanisms mediate these changes and later outcomes in terms of selection. The combination of the above research areas gave rise to the field of ecological immunology (ecoimmunology), which primary goals are to understand the factors underneath immune investment [2] variation across a wide range of animal species, throughout annual-cycles, upon different environments and under an evolutionary perspective and how these changes contribute to disease susceptibility (Sheldon and Verhulst 1996; Norris and Evans 2000; Lee 2006; Sadd and Schmid-Hempel 2008; Hasselquist and Nilsson 2012). Therefore, ecoimmunologists must undergo broadly integrative research studies, incorporating subjects like ecology, life-history theory, evolution, endocrinology, molecular biology and behaviour to address the new questions posed by this recent research area.

[1] The inability to resist infection (Schmid-Hempel 2011).
[2] Term is loosely used to refer to the capacity of an individual respond to a pathogen challenge, keeping in mind the immune system complexity and that response operates in different though highly correlated broad axis (Schmid-Hempel 2011).

# The Costs of Immunity

One of the founder ideas of ecoimmunology is that immunity comes with costs, as well as benefits, and that trade-offs are generated within different energy competing physiological mechanisms (Sheldon and Verhulst 1996). This leads to differences in "how much" energy that should be devoted to immunity at specific situations (or life-stage) of an animal's life. Energy is a limited resource, therefore allocation towards different aspects of an animal annual-cycle is constantly undertaken. However, from the ecological point of view, conflicts arise when one physiological activity competes with others for the same resources, for example, maintenance of the immune machinery [3] and activation of certain immune responses, incurs costs and consequently a trade-off with other physiological and/or behavioural processes are expected to occur (*e.g.* breeding, migration, growth) (Fig. 1; Sheldon and Verhulst 1996; Norris and Evans 2000; Lee 2006; Hasselquist and Nilsson 2012). The individual's capacity to fight and control infections has obvious benefits, being therefore an essential mechanism for self-maintenance. The inability to do it, or delivery of an inadequate response (too strong or too weak), can lead either to collateral tissue damage and development of autoimmunity complications, or do not prevent infection from spreading. On both situations, negative effects of immune response can lead to fitness reductions or in more extreme cases, to death (Råberg et al. 1998; Lochmiller and Deerenberg 2000; Norris and Evans 2000; Råberg et al. 2002). Consequently, the optimal immune response [4] is hypothesized to be one of the driving selective pressures at the individual level (Lochmiller and Deerenberg 2000; Ricklefs and Wikelski 2002; Schmid-Hempel 2003; Sadd and Schmid-Hempel 2008).

[3] Summarizes the molecular, biochemical and physiological processes that make immune response.
[4] Theoretical combination of different immune system components and/or the strength of response that yields the highest fitness, taking into account it´s costs and benefits (Schmid-Hempel 2003; Tschirren and Richner 2006).

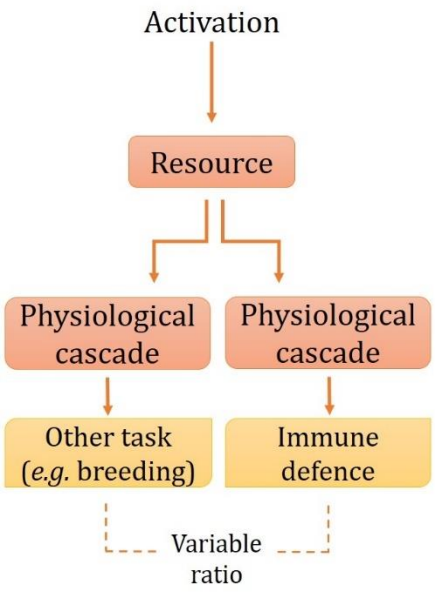



**Fig. 1 –** Schematics of the trade-off for resource allocation. Immunity like other physiological components requires a steady supply of resources (or energy) to function. Under an energetic limited system, the amount of resources allocated to a wide variety of physiological functions is constraint and traded-off at variable rates within other functions to maximize fitness. The total amount of resources allocated to immunity is thus dependent upon the available energy (*e.g.* access to food) as well as the energy expenditure required at a specific life-stage (*e.g*. moult) and environmental context (*e.g.* thermoregulation). Schematics adapted and modified from Schmid-Hempel 2011.

When it comes to "costs", they are not only measured in terms of the immediate nutritional costs which are ultimately transformed in energy (also called *resource costs*), but also in other physiological components that have a much broader meaning (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003; Hasselquist and Nilsson 2012). The most basic cost is the *evolutionary cost*, in which the immune system evolved at the expense of another trait. In other words, it means that one selective advantage of an increased immune function is unavoidably correlated with a loss of other fitness component (Schmid-Hempel 2003, 2011). Most of the findings that support this notion come from studies in invertebrates, such as insects with less complex immune systems. For example, the increased resistance of mosquitoes to nematode infections leads to losses in terms of reproductive success (Ferdig et al. 1993). Another cost is the *immunopathology cost* which is paid by the collateral damages caused by an over reactive immune system, *i.e.* when the immune system attacks

own cells and proteins (self-reactivity) (Råberg et al. 1998; Hasselquist and Nilsson 2012). Finally, there are *opportunity costs* which translate into lost opportunities in terms of time devoted to mating, migration and moult caused by allocation of resources towards immune responses (Owen-Ashley and Wingfield 2007). Evidences of this type of costs come from activation of costly immune responses, that lead to temporary (short or long-term) suspensions of other life-stage activities, like breeding, feeding opportunities, migration delays, or return-rates (*e.g.* Hanssen et al. 2005; Råberg et al. 2000; Gasparini et al. 2009). For example, when female Pied flycatchers (*Ficedula hypoleuca*) were immune challenged by an injection of a tetanus vaccine, the activation of the immune system had measurable and negative consequences over other fitness components such as foraging effort and number of offspring (Ilmonen et al. 2000).

Besides the *cost prediction axis*, another immunity axis has been put forward to explain variation in immune investment. This axis is based on immune investments in relation to the environmental pathogen risk that birds are experiencing. Subsequently, investments should be greater in areas/habitats where pathogen pressure is higher (Zuk and Stoehr 2002; Lindström et al. 2004; Tschirren and Richner 2006; Horrocks et al. 2011). In these particular cases, the cons of building a strong immune response and risk collateral tissue damage are balanced by the maximization of pathogen elimination, which is a necessary strategy to maximize survival and enhance fitness in these type of habitats. Despite the existence of habitat and environmental gradients in parasite abundance (Figuerola 1999; Mendes et al. 2005; Salkeld et al. 2008; Clark et al. 2015), quantified measures of this variable is unarguably hard to obtain in complex ecosystems, and there is limited research using natural bird populations as study-models.

On a broader perspective, the myriad of life-history strategies that had evolved to maximize survival and reproductive success, most likely lead to an immense variety of immune response strategies, creating difficulties to make clear predictions about immune investments (Ricklefs and Wikelski 2002). The costs of immune response and trade-offs are often difficult to assess correctly in free-living birds, for a number of reasons. So far, several studies have focused on understanding trade-offs between immune investments and other life-history strategies, namely breeding (reviewed in Knowles et al. 2009), mating efforts

(Kilpimaa et al. 2004), moult (Buehler et al. 2008a; Moreno-Rueda 2010), migration (Hasselquist et al. 2007; Nebel et al. 2012; Eikenaar and Hegemann 2016), stress (Matson et al. 2006a; Buehler et al. 2008b), hormones (Hasselquist et al. 1999) and workload (Hegemann et al. 2013a). Nonetheless, most studies represent only a small window of a wider annual cycle and few had measure immune variation throughout seasons and/or during multiple years of a given species (Owen-Ashley and Wingfield 2006; Horrocks and Matson 2012; Hegemann et al. 2012a; Hegemann et al. 2012b; Versteegh et al. 2014). Even fewer studies had taken into account one of the most important triggers and modulation factors of immune investment, the exposure to pathogens (Horrocks 2012; Horrocks et al. 2011; Horrocks et al. 2012). A good framework to assess the complex outcome of immune responses and disease susceptibility should minimize interference of confounding factors like genetics, life-history strategies, ecology and host parasite co-evolution.

## Migration and Disease

The obvious benefits of migration come from taking advantage of typically high productivity areas, which normally also have less predators and parasite pressure (Alerstam et al. 2003). An example of such areas is the (low) Arctic, in which the brief summer under a continuous daylight give origin to relatively high productivity plant communities that are readily available for herbivores (Smetacek and Nicol 2005), such as geese that migrate to these locations and graze in large concentrations in the arctic tundra. However, their journeys also come with costs and risks, not only because they face constantly changing abiotic and biotic factors (Newton 2008), but also because migration by its own right is energetically costly at the physiological, nutritional and immunological level (Piersma and Gill 1998; Piersma et al. 1999; Nebel et al. 2012; Alves et al. 2013). Just before the migratory periods, birds undergo physiological and metabolic adjustments, including changes in size and/or activity of some organs (some related to immunity), to accommodate the strenuous long-distance flights (Piersma and Gill 1998; Piersma et al. 1999). In addition, the migratory restlessness triggered by stress hormones, actively suppress the immune system, leading birds more vulnerable to infections. This was the mechanism behind the reactivation of *Borrelia* spp.

infections (Lyme disease) on captive redwing thrushes (*Turdus iliacus*) (Gylfe et al. 2000). Depending on the animal study system, the "costs of migration" generally come with wider exposure to pathogens. On a global scale, the abundance and diversity of pathogens increases when latitude decreases, meaning that infection risk is higher in the tropics than in temperate regions (Guegan et al. 2008; Salkeld et al. 2008). Therefore, compared to resident temperate region birds species that remain most of their lives in the same areas, migrants that move between temperate and tropical regions are naturally exposed to different parasite communities along their flyways, so it is likely that they had evolved unique strategies to cope with pathogens and withstand their selection pressures (Møller and Erritzøe 1998; Møller et al. 2003; Hasselquist 2007; Altizer et al. 2011; Westerdahl et al. 2014). At the same time, migrants also tend to adopt high-risk behaviours that may favour transmission of certain kinds of pathogens (Altizer et al. 2011). For example, the mixed species high density flocks typical of waders when on stop-over or wintering sites (Fig. 2; Newton 2008), can be "hot spots" for the transmission of avian influenza virus, both within and between species (Krauss et al. 2010). The existence of larger and/or heavier immune system organs (spleen and bursa de Fabricius) in migrants is also an evidence for the increased risk of contracting infections, and their capacity to build stronger immune responses (Møller and Erritzøe 1998; Ardia 2005). Migrants also play key-roles for the worldwide distributions of certain pathogens, like virus (*e.g*. avian influenza virus), bacteria (*e.g.* Lyme disease) and protozoan parasites (*e.g.* avian haemosporidians) (reviewed in Hubálek 2004).

Cumulating evidences coming from various animal systems shows that strenuous work, directly or indirectly, lead to negative effects over the immune system, thereby exposing animals more vulnerable to disease during specific periods of their annual cycles. Latitudinal and habitat-related differences on pathogen pressure (Figuerola 1999; Mendes et al. 2005; Yohannes et al. 2009; Clark et al. 2015) is therefore hypothesized as one of the reasons for the existence of two co-evolutionary migratory strategies in long-distance migrant waders, in which high arctic breeders are restricted to marine/coastal habitats (hence more devoid of pathogens); while temperate breeders use freshwater/inland habitats outside the breeding season (Piersma 1997, 2003). This hypothesis may help

understand the physiological trade-offs between the 10,000 km non-stop migration flights undertaken by Bar-tailed godwits, only stopping at relative parasite-free areas (Gill et al. 2009), sparing individuals from high investments in immune response and redirecting energy towards these amazing tasks.

**Fig. 2 –** During spring migration high density flocks that can reach 40,000 individuals of Black-tailed godwits, congregate on the rice-fields of Tagus estuary. Photo by Sara Pardal

## The Immune System

The immune system of mammals and birds, originate from our common reptilian ancestor 200 million years ago, and protects us from infectious parasites[5] (*e.g.* viruses, bacteria, fungi, protozoa, parasitic worms) that inhabit our world (Murphy and Weaver 2017). Despite that many bacteria are found as commensal microbiota in our gut, the immune system needs to check these organisms and their growth since they may become non-symbiotic, and even harmful or highly pathogenic (Hooper et al. 2012). Our immune system has evolved complex

[5] Term pathogen and parasite will be used interchangeably to define, in a broad sense, infectious agents.

and remarkable strategies to prevent infection, exploitation and disruption of physiological and biochemical systems, and it is essential for our survival (Zimmerman et al. 2010).

The immune system includes all the structures and processes that provide a defence against potential pathogens. The intricate immune responses have been described in many different ways, but one of the most comprehensive is to divide the immune responses in two broad axes: *innate* (nonspecific) and *adaptive* (or specific) immunity, and *constitutive* (inherited as part of the structure of an organism and therefore constantly maintained) and *induced* (triggered by the presence of a pathogen), respectively (Janeway and Medzhitov 2002; Lee 2006; Zimmerman et al. 2010; Murphy and Weaver 2017). Classically, an innate response is considered important on the initial stages of infection, limiting the spread of novel infections until development of an adaptive response. The immune responses of innate immunity occur within a day, whereas the adaptive responses take longer, more than a week. The innate immunity recognizes structures that are unique to pathogens and the ability of the innate immunity to differentiate between pathogens is low (less specific) compared to adaptive immunity. Another important characteristic distinguishing innate and adaptive responses is the immunological memory. While an adaptive response is much more efficient and faster on a second exposure to the same pathogen, the magnitude of an innate response is not influenced by a prior exposure and it simply reflects a response to an immediate stimulus (Juul-Madsen et al. 2008). In conclusion, innate immunity is the first line of defence against pathogens and it also triggers adaptive immunity. Adaptive immunity is mediated by lymphocytes (T-cells and B-cells), it takes time to become activated but it is highly specific and has immunological memory (Murphy and Weaver 2017).

## Barriers and types of defences

Pre-infection defences actually start outside and not inside the body, and they act to minimize exposure and reduce risks of successful entry by physical (*e.g.* skin) or chemical (strong pH differences) barriers. Though, avoidance of infection can also be behavioural through preening or grooming, or just by avoidance of certain diets, places (spatial

avoidance) or certain times of the day or seasons (temporal avoidance) (Schmid-Hempel 2011). In some animal systems, migration could also be viewed has a spatial and temporal avoidance strategy, since animals avoid infection by moving away from those risky areas and/or seasons (Schmid-Hempel 2011).

However, when pathogens do overcome these first barriers and enter the body, innate immunological defences are the first employed to control the infection (Fig. 3) (Zimmerman et al. 2010; Juul-Madsen et al. 2008). Immune system surveillance cells such as heterophils and macrophages, attack extracellular pathogens such as bacteria or parasitic worms (arrow 1; Fig. 3), by engulfing these pathogens and producing soluble mediator molecules that attract more phagocytes to the site of infection (Juul-Madsen et al. 2008). In the case of invasion by intracellular pathogens, such as virus, natural killer cells will be deployed instead. If these defences are not enough to deter the invaders, macrophages release cytokines that induce the acute phase response (arrow 2; Fig 3), with the accompanying symptoms of fever, lethargy (*i.e.* inactivity) and anorexia (*i.e.* lack or/loss of appetite) (Owen-Ashley and Wingfield 2007). Activation of this mechanism in severe infections, though having a beneficial function, such as inhibiting bacterial activity, it also diverts essential amino acids from other organs (mostly muscles) and away from other physiological components leading to significant reductions in body mass (*e.g.* 15-30% in humans) (Long 1977) and increasing resting metabolic rates (*e.g.* 25-55% in humans) (Kreymann et al. 1993). This is because nearly every defence mechanism require great amounts of amino acids for protein production (Lochmiller and Deerenberg 2000; Owen-Ashley and Wingfield 2007). The innate immune response also has the ability to trigger an adaptive immune response. All nucleated cells present own and foreign material (as peptides on MHC class I receptors) to patrolling cytotoxic T-cells from the adaptive immunity, while antigen presenting cells, like dendritic cells, that have engulfed pathogens present pathogen peptides (through MHC class II receptors) to helper T-cells (arrow 3; Fig. 3). Dendritic cells can also present peptides from pathogens engulfed by other cells (such as macrophages), by so called cross-presentation. Moreover, dendritic cells also have the ability to activate naïve T-cells, here dendritic cells that have taken up pathogens migrate to the nearest lymph node where they activate T-cells that proliferate and are ready to eliminate pathogens. If the

pathogen is intracellular (like viruses and some bacteria) there will be a cell-mediated response and if the attacker is an extracellular pathogen, then an antibody-mediated response will be triggered based on antibodies via B-cells (arrow 4; Fig. 3) (Kaiser and Stäheli 2008; Penn and Potts 1999). The antibodies and cytokines will feedback into the non-specific surveillance cells, significantly increasing the destruction through phagocytosis (arrow 5; Fig. 3) by specifically marking the attackers. When the infection is cleared, T and B-cells with specific receptors for the particular pathogen will remain as memory cells, providing a more immediate action in case of reinfection by the same pathogen. For more detailed information, see Box 1.

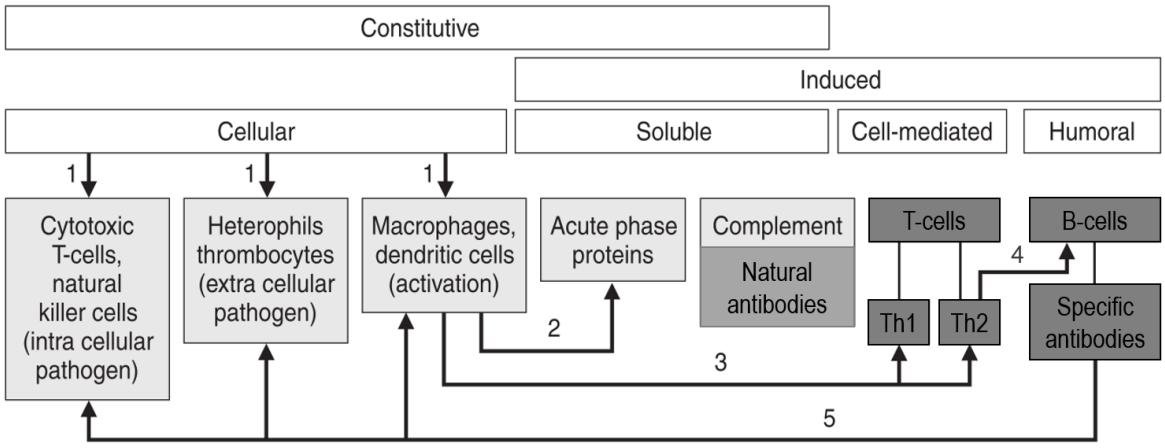



**Fig. 3 –** Simplified diagram of the avian immune system. Immune surveillance cells and soluble mediators are shown below each category, while shading represents the specificity of each component. Light-grey shading represents low specificity innate immunity, and dark-grey shading represents high specificity adaptive immunity. Natural antibodies have an intermediate shading due to their broad specificity. Arrows indicate a simplified path of the pathogens and steps taken by the immune system (see text). White boxes represent one of the axes that divide the immune system, the constitutive immunity that is constantly maintained and the induced immunity that is triggered after pathogen exposure. Cellular and soluble components are immunity mediators found on body fluids, while cell-mediated and humoral-mediated immunity indicate a response either undertaken by T-cell or antibodies produced by B-cells. Figure adapted and modified from Buehler (2008).

## Differential immunity costs

The immune system has many layers and each component comes with its own benefits and costs. Evidences point to differential costs of maintenance[6] and deployment[7] of innate and adaptive responses (Ots et al. 2001; Martin et al. 2003; Klasing 2004; Lee 2006). Innate constitutive defences has low development and moderate maintenance costs, since the existent leukocyte (excluding lymphocytes) cells do not require any kind of selection/diversification, because they are long-lived (except for heterophils) and the vast majority are "at rest" in a healthy individual (Klasing 2004). However, activation of the induced innate immunity such as the acute phase response, the accompanying symptoms of fever, anorexia and lethargy are extremely costly not only at the energetic and nutritional level (Lochmiller and Deerenberg 2000; Owen-Ashley and Wingfield 2007; Hegemann et al. 2012b), but also have a carry-over effect over behaviour and fitness (Wobeser 2006). The indirect effects of lethargy and anorexia can lead to increased susceptibility to predation, accidents, reduced competitiveness and nutritional stress (Lochmiller & Deerenberg 2000; Wobeser 2006). On the other hand, constitutive adaptive defences present very high development costs from the embryonic stage until chicks reach 4 weeks old (Klasing 2004). The reason is mostly because the receptors that lymphocytes use to recognize pathogens are generated by a series of semi-random combinations and point mutations within the thymus and bursa of Fabricius (in birds) and therefore most of the produced T and B-cells are ineffective (Klasing 2004; Lee 2006). Functional antigen recognition sites, in chickens for example, represent only 5-10% of the total produced cells, where the surplus must be discarded (Klasing 2004). Nonetheless, once developed and because of the existence of immunologic memory, maintenance and use of the adaptive immunity is metabolically cheaper that due to the high specificity, the risk of self-damage to tissues is quite low (Råberg et al. 2002; Klasing 2004).

[6] Defined by the "steady-state" levels of surveillance cells and proteins engaged on their routine functions (Klasing 2004).
[7] Defined by a response towards a pathogen.

## The key-genes of adaptive immunity

The major histocompatibility complex (MHC) holds the most polymorphic genes known, and the MHC genes encode cell surface receptors (glycoprotein´s) whose function are tightly linked to antigen recognition and activation of adaptive immune responses (Kaufman 2008). MHC receptors are involved in antigen presentation to T-cells, mediating interaction between lymphocytes and other leukocytes and body cells (Hess and Edwards 2002; Kaufman 2008). The antigen binding sites (or peptide binding sites) are therefore under selective pressure by the parasites they recognize, and these selective traces can leave genetic footprints in the host genes (Westerdahl et al. 2014). At the same time, the same selective pressures also shape parasite genes, leading to constant (re)adaptations which, on an evolutionary scale, can be translated into a host-parasite coevolution (Schmid-Hempel 2011). MHC genes are therefore particularly relevant for molecular adaptation studies in vertebrates, as they can be a reflection of evolutionary and adaptive processes within and among populations (Sommer 2005).

## MHC classes and antigen recognition

MHC genes exist in a multigene family arranged into three different classes: class I, class II and class III, according to their function and antigen presentation within the adaptive immune response (Hess and Edwards 2002). Though MHC receptors class I and II have a similar overall structure, the domains are connected differently and function in dissimilar ways by recognizing intracellular (mostly virus and some bacteria) and extracellular (bacteria, nematodes, cestodes) pathogens, respectively, while class III make up mainly the complement component (Hess and Edwards 2002; Sommer 2005).

Classical MHC *class I* glycoprotein's receptors are found on almost all cells and bind peptides derived from endogenous proteins in the cytoplasm or nucleus (Kaufman 2008). Endogenous proteins include host´s own proteins, tumour antigens or pathogen derived antigens (Kaspers et al. 2008). If the peptide is the product of foreign or transformed antigen, the antigen-MHC complex can then be recognized by $CD8^+$ T-cells, cytotoxic T-cells. This

recognition activates and induces $CD8^+$ cytotoxic cells to proliferate and destroy cells presenting the same antigen on their surface (Kaufman 2008). Activated cytotoxic T-cells have consequently the capacity to kill virus and/or bacterial infected or transformed cells (tumour cells) (Kaufman 2008; Kaspers et al. 2008).

Classical MHC *class II* receptors are only found on specialized antigen-presenting cells, such as macrophages, dendritic cells and B-cells (Hess and Edwards 2002; Kaufman 2008). Binding of MHC molecules with foreign or transformed antigens is made either from intracellular vesicles derived from the phagocytosis made by macrophages, or by taken up from the extracellular space by dendritic cells and B-cells (Kaufman 2008). After the antigen-MHC complex is formed on these intracellular vesicles, they are transported and presented on the cells surface to be recognized by $CD4^+$ T-cells, normally helper T-cells. Helper T-cells will induce an immune response against extracellular pathogens, by stimulating B-cells for the production of antibodies and macrophages to kill the same pathogen (Kaufman 2008). In conclusion, peptide presentation by MHC proteins is a crucial step for the triggering of T-cells and an adaptive immune response.

## MHC mechanisms of selection

The unusual diversity of MHC across natural systems cannot be explained by normal processes of neutral evolution, but rather to some form of balancing selection (Hess and Edwards 2002; Sommer 2005; Spurgin and Richardson 2010). Balancing selection includes several forms of selection that act to maintain multiple alleles within populations (reviewed in Spurgin and Richardson 2010). Several of these selection mechanisms have been proposed, but MHC codes for the recognition of molecules which bind to pathogen derived peptides, so pathogen-mediated selection (PMS) is suspected to be one of the main drivers (reviewed most recently by Piertney and Oliver 2006; Spurgin and Richardson 2010). We can therefore expect a correlation between high PMS and specific MHC alleles or MHC diversity (Piertney and Oliver 2006; Spurgin and Richardson 2010). Aside from PMS mechanisms, non-pathogen mechanisms are also responsible for shaping MHC evolution, such as gene conversion, recombination, duplication, sexual selection, autoimmunity and kin

recognition (Edwards and Hedrick 1998; Martinsohn et al. 1999; Penn and Potts 1999; Balakrishnan et al. 2010). Most importantly is that none of the described mechanisms are mutually exclusive, and MHC diversity can be maintained by a combination of factors (Sommer 2005).

## *Pathogen-mediated selection: an "arms-race"*

Pathogens are likely to drive MHC diversity because certain alleles determine susceptibility or resistance to a specific parasite, influencing their long-term survival (Piertney and Oliver 2006; Spurgin and Richardson 2010). Currently, three main types of balancing selection are proposed: *heterozygote advantage*, *negative frequency-dependent selection* and *fluctuating selection* (Doherty and Zinkernagel 1975; Takahata and Nei 1990; Hedrick 2002).

According to the *heterozygote advantage* hypothesis, heterozygous individuals at the MHC loci are more able to detect a wider range of pathogen peptides than homozygous, and consequently benefit from increased resistance (Doherty and Zinkernagel 1975). Heterozygous individuals are therefore expected to benefit from higher fitness, because having more different alleles will increase the chances for a specific antigenic peptide to be recognized by T-cells, especially if confronted with multiple strains of pathogens (Sommer 2005; Milinski 2006; Spurgin and Richardson 2010). This characteristic can be especially advantageous to migratory birds, because they contact with larger parasite communities during their migrations. Heterozygosity advantage effects on disease resistance have been described for fish, humans, sea lions and birds (Thursz et al. 1997; Carrington et al. 1999; Senseney et al. 2000; Arkush et al. 2002; Osborne et al. 2015), though some studies also point to association of specific alleles to disease resistance (*e.g.* Hill et al. 1991; Westerdahl et al. 2012) or intermediate levels of heterozygosity favouring fitness (Wegner et al. 2003; Bonneaud et al. 2004).

The *frequency-dependent selection* (or rare-allele advantage) occurs under a dynamic interaction of hosts and parasites. This co-evolutionary arms-race assumes that pathogens will try to overcome host defence, and hosts will try to reduce pathogen infection and exploitation (Bodmer 1972; Takahata and Nei 1990). Pathogen resistance to the most

common MHC alleles will provide a selective advantage to individuals carrying rare alleles that confer resistance, but this advantage will only last as long as the pathogen have not adapted to it. A consequence of this pathogen-driven interaction will be a fluctuating and cyclical frequency selection of specific alleles preventing them from becoming fixed or eliminated in the population (Spurgin and Richardson 2010).

The last hypothesis *fluctuating selection* underlines that pathogens not only vary on frequency and intensity, but also have temporal and spatial patterns. Thus selection of certain MHC alleles will vary on a time scale and also among habitats/environments within the range of a species (Hedrick 2002; Sommer 2005). Local adaptation to different habitat and subsequent parasitic faunas, can explain the observed differences on MHC composition of mole rats (*Spalax ehrenbergi*) (Nevo and Beiles 1991) and great snipes (*Gallinago media*) (Ekblom et al. 2007).

## *What lies behind sexual selection?*

Sexual selection in birds is a particularly notorious evolutionary force, which lead to some extreme ornamentation, colouration and elaborated courtship´s dances, such as those exhibited by birds of paradise (Grether 2010). Due to the strong selective pressure of parasites in the natural environment, it has been hypothesized that some secondary sexual characteristics are linked to immune responsiveness and subsequently that animals should choose their mates based on disease resistance (Hamilton and Zuk 1982; Hamilton et al.1990). Sexual signals should be reliable quality indicators, because parasitic infections and immune up-regulation divert energy away from breeding investments and have a direct impact on host condition, which in turn influences the expression of the signal and thus mate selection (Sheldon and Verhulst 1996; Zuk and Stoehr 2002; Maan and Seehausen 2011). Parasitic exposure may also vary according to habitat (*e.g.* lake *vs.* river habitats in fishes; Scharsack et al. 2007) and dietary resources (*e.g.* different prey items in mice; Luong et al. 2013) and sexual signals may convey information on how individuals make use of the surrounding environment (Maan and Seehausen 2011). Lastly, parasites evolve more rapidly than hosts, and differences in mate choice will help to maintain the necessary genetic

variation, providing a moving target (*i.e.* the Red Queen Hypothesis) to parasites that evade immune recognition (Ebert and Hamilton 1996; Milinski 2006). The trade-offs between parasitic resistance and sexual secondary characters related costs provides a selective resistance filter, preventing cheating and may explain why females have evolved a preference for mates that exhibit high quality secondary sexual characters (Grafen 1990). If these signals are used as indicators of mating preferences there must be individual variation, and in fact, many studies found a connection between immune capacity and male secondary sexual characters such as the long tails of swallows (Saino et al. 1997), song complexity (Duffy and Ball 2002; Garamszegi et al. 2003), bill colour intensity (Blount et al. 2003; Baeta et al. 2008) and bright coloration of passerines (Trigo and Mota 2015). In conclusion, given that mate choice may often serve as an "amplifier" of pathogen-mediated selection and that sexual secondary characters act as "honest" signals of male quality, correlations between male secondary sexual characters and innate and/or adaptive immunity parameters, are therefore likely to occur in waders and in their diverse mating strategies (Székely et al. 2006).

## Measuring Innate and Adaptive Immunity

Measuring immune function is a reliable tool, because it can help us not only to make inferences about pathogen pressure (Horrocks 2012), but also differences in the ability to fight infection (Møller and Erritzøe 1998; Ardia 2005). In addition, as a costly component, measures of the immune function can provide inferences of self-maintenance investments in the context of ecoimmunology (Sheldon and Verhulst 1996; Sadd and Schmid-Hempel 2008; Ardia and Schat 2008; Møller and Saino 2004). For species like long-distance migrants that encounter a wide range of pathogens along their annual cycle (Møller and Erritzøe 1998), innate immunity becomes important and relevant from the evolutionary perspective, because it is a quick first line of defence against a broad scope of pathogens (Buehler 2008; Juul-Madsen et al. 2008). Subsequently, since innate immunity has no immunological memory, basal levels can be measured at a single point (*e.g.* in one bird capture) and response remains the same irrespective of prior exposure to the same or new pathogens

(Buehler 2008; Juul-Madsen et al. 2008). Hence, measuring this branch of immunity become a useful quantitative marker of exposure to pathogens, and is ideal for studies in free-living birds. To understand interactions and evaluate individual immune system capacity at the time of assessment (single-time point measurements) the chosen immune assays in this study were: heterophils/lymphocytes (H/L) index, leukocyte counts (Harmon 1998; reviewed in Davis et al. 2008), hemolysis-haemagglutination assay (Matson et al. 2005) and haptoglobin (Matson et al. 2012a; Matson et al. 2006b). For more detailed information about the utility of the assays used in this thesis, please see Box 2.

As a measure of adaptive long lasting immunity, characterization and deep sequencing of MHC genes, required Sanger sequencing as well as the recently developed high-throughput sequencing Illumina technology (Reuter et al. 2015). Both tools will allow us to perform a large scale characterization study of these genes on our study-species and provide us relevant information of the evolutionary and selective pressures in different environments.

# BOX 1. IMMUNE SYSTEM MEDIATORS

CELLULAR MEDIATORS

### *Leukocytes*

Five types of leukocytes (or white blood cells) can be found within the bird's blood: heterophils (the avian correspondent to mammalian neutrophils), eosinophils, basophils, lymphocytes and monocytes. Since heterophils, eosinophils and basophils contain a lobed nuclei and cytoplasmic granules, they are collectively referred as *polymorphonuclear granulocytes*. Lymphocytes and monocytes on the other hand are *mononuclear cells* (Clark et al. 2009). Regarding function, leukocytes can be divided into two main categories: phagocytes and lymphocytes. Phagocytes main function is to ingest and destroy foreign substances or apoptotic and dyeing cells. Their "professional" phagocytic cell is the *macrophage* which is a long-lived cell, while his precursor the *monocyte*, circulates throughout the body providing surveillance (Kaspers et al. 2008). The polymorphonuclear granulocytes are also phagocytic cells, in which the *heterophils* unlike the macrophages are short-lived cells whose main function is to phagocytose and destroy pathogens and induce the expression of several pro-inflammatory cytokines. These cells proliferate in circulating blood and during the early stages of infection are recruited to the surrounding tissues, so their circulating levels become reliable indicators of not only current inflammatory status, but also stress and infection (Davis et al. 2008). *Eosinophils* are cytotoxic cells, far less common than heterophils, which main role is to kill other cells as well as parasitic worms (*e.g.* helminth parasites) and are also involved in inflammatory process. *Basophils* are the rarest cells whose function is still not clearly understood, but thought to also mediate inflammation (Davis et al. 2008).

Lymphocytes, are produced and developed in lymphoid organs, the thymus and bursa de Fabricius (or bone marrow in mammals) and are specialized cells of the adaptive immunity (Murphy and Weaver 2017; Davison et al. 2008). These cells occur in two major types and named depending on their differentiation sites and antigen receptors: B-cells (when originated in the bursa) and T-cells (when originated in the thymus) (Davison et al. 2008). The B-cells fight extracellular

pathogens through the production of specific antibodies into the blood and lymph, providing *humoral immunity* and therefore essential on *antibody-mediated immunity* mechanisms. As soon as a B-cell encounters a specific pathogen, it becomes activated and multiplies and differentiates into a plasma cell, capable of producing large amounts of antibodies which will mark and greatly increase the efficiency and specificity of phagocytosis (Murphy and Weaver 2017). When infection is cleared, these cells will remain as long-lasting memory cells, providing quick and specific protection in case of reinfection (Murphy and Weaver 2017). On the other hand, the T-cells (specifically cytotoxic T-cells) recognize digested non self-particles and attack infected host cells or cancerous cells. Recognition is made by specialized major histocompatibility complex (MHC) receptors and since T-cells need to physically contact the cells to trigger a response, they are become key on *cell-mediated immunity* mechanisms. The ability for the recognition of large pathogens repertoires by these cells, relies subsequently on the high diversity of T and B-cells (Murphy and Weaver 2017).

SOLUBLE MEDIATORS

### *Complement system*

Refers to a series of serum proteins, cell surface receptors and regulatory proteins that circulate in the blood in their inactive form and are involved in inflammatory processes. In response to pathogen stimuli, these proteins are activated in cascade, enhancing the phagocytosis of pathogens by opsonization (coating), inducing an inflammatory response by attracting B and T-cells to the site of infection and finally by direct killing of infected cells or gram-negative bacteria by lysis of their cell membranes (Ochsenbein & Zinkernagel 2000; Juul-Madsen et al. 2008). In cooperation with natural antibodies, the complement becomes an important first line defence against infection (Thornton et al. 1994).

### *Antibodies*

Antibodies, also known as immunoglobulins (Ig), are glycoproteins produced by B-cells which are highly specific and can be found in the blood, lymph and vascularized tissues (Murphy and Weaver 2017). Natural antibodies (NAb) are a special kind of

immunoglobulins (mainly IgM, although IgG and IgA had already been found) with a broad specificity but low binding affinities, involved in the clearance of foreign, dead or catabolic materials, enhancing antigen uptake and processing and presentation by B-cells or dendritic cells (Davison et al. 2008; Ochsenbein et al. 1999; Ochsenbein & Zinkernagel 2000). They differ from the induced antibodies in the sense that they their levels do not depend of pathogen stimuli, but are spontaneously segregated by B-cells and therefore constantly maintained (Ochsenbein et al. 1999).

### *Acute phase proteins*

Acute phase proteins are defined as those whose plasma concentrations change dramatically after bacterial infection, injuries (*e.g.* bone fractures), trauma and inflammatory processes. Members of these group include among others, the C-reactive protein, fibrinogen and haptoglobin that are involved in three major biological functions: participation on host defence mechanism, inhibition of harmful products that result from pathogen invasion and transport of proteins with antioxidant activity (Juul-Madsen et al. 2008).

# BOX 2. CONSTITUTIVE INNATE IMMUNITY ASSAYS

LEUKOCYTE COUNTS

Five types of leukocytes are encountered in the bird's blood, namely heterophils, eosinophils, basophils, lymphocytes and monocytes (Clark et al. 2009). Leukocyte concentration provide information on circulating immune cells which can be an indicator of health (Harmon 1998; Davis et al. 2008). Heterophils and eosinophils, are granulated leukocytes and the primary phagocytic leukocytes, therefore important mediators of innate immunity that proliferate in circulation as a response to inflammation, infection and stress (Harmon 1998; Davis et al. 2008). Monocytes also have a phagocytic activity and their cytoplasmic lysozymes are involved on mediating inflammation, being a link between innate and acquired immunity (Mitchell and Johns 2008). Basophils are the rarest type of leukocyte cells and are thought to be involved in inflammation (Murphy and Weaver 2017). In birds, eosinophils together with lymphocytes are the most common circulating leukocytes (Clark et al. 2009) and during the course of an infection, inflammation or stress, the ratios of both cells vary in opposite directions (Davis et al. 2008). In these cases, the heterophil numbers increase rapidly in the blood, while lymphocytes are recruited from circulation and into other tissues, becoming H/L ratios a reliable marker for above mentioned processes.

HEMOLYSIS-HAEMAGGLUTINATION ASSAY

Assay used to access immune responsiveness, namely by quantifying complement and natural antibodies activity (Matson et al. 2005). The complement cascade and natural antibodies are considered the first line defence of innate immune system (reviewed in Ochsenbein & Zinkernagel 2000, Juul-Madsen et al. 2008). They are also involved in the clearance of foreign, dead and catabolic materials, enhancing antigen uptake and processing and presentation by B-cells or dendritic cells (Thornton et al. 1994; Juul-Madsen et al. 2008). The assay consists on mixing rabbit red blood cells (RBC) with serial dilutions of bird's plasma. Hemolysis indicates the amount of haemoglobin released from lysed rabbit RBC as a result of complement´s lytic activity, while haemagglutination reflects the action of

natural antibodies (NAb) (Matson et al. 2005).

HAPTOGLOBIN

Haptoglobin is an acute phase protein that binds free haemoglobulin (iron), which is toxic, pro-inflammatory and used as nutrient for pathogens. It also offers protection against harmful end products (oxidative stress) of the immune response (Juul-Madsen et al. 2008). Elevated levels of haptoglobin indicate current infection, inflammation or trauma (Matson et al. 2012a). Several commercial kits are available for measuring the levels of the peroxidase activity of haptoglobin when bounding to haemoglobulin.

# Thesis Outline

Pathogens play a prominent role upon natural selection and on an evolutionary scale, and species have shaped the optimal immune defence for their annual cycle and accordingly to the disease landscapes they inhabit. The main goal of this thesis is to give a comprehensive understanding of how environmental pathogen pressure modulates immune strategies at the innate and adaptive level, and if and how other drivers like the annual cycle stages and sexual selection are mediators of the individual´s immune responsiveness. For that I collected data from two different subspecies of a long-distance migratory wader, the Black-tailed godwit (*Limosa limosa*) in which their migratory strategies impose a differential exposure to pathogens, especially vector-borne. Biological samples were collected at specific stages of the species annual-cycle, comprising the breeding, autumn and spring migration periods, as well as the wintering periods. Thus the data presented in this thesis was gathered not only across a latitudinal gradient from the subarctic (Iceland) to the tropical areas (Guinea-Bissau), but also collected in individuals that occupy different wetland habitats (freshwater *vs.* brackish water).

This thesis is an original contribution for the understanding of the factors underneath immune investment variation upon: 1) different environments and life-stages; 2) under an evolutionary perspective; and 3) mate choice and sexual secondary characters as signals of the capacity to fight infection. We also 4) characterize for the first time the MHC class I gene for the study species, which becomes relevant for MHC broader comparative macro-evolutionary studies across bird communities and 5) provide a comprehensive methodological approach for the effects of plasma samples storage upon the final outcome on several of the most commonly immunological assays used nowadays to test hypothesis on the ecoimmunology and evolutionary context. To achieve the above goals, the following studies were undertaken:

**Chapter I -** To address the effects of environmental pathogen-pressure on the immune system, we assessed the investment on baseline constitutive immunity components along the flyway of two wader subspecies that are genetically similar, but vary on migratory strategies and habitat-use. This work contributes to understand the drivers of baseline immunity and physiological trade-offs along the flyway, and is the first to show a clearer effect of pathogen pressure modulation on the innate immune system.

**Chapter II -** MHC characterization studies have been mostly restricted to passerines, while in other bird groups information remains incomplete or even absent, and hence limiting the potential for broader comparative analyses. We therefore set out to firstly partly characterize MHC-I, and secondly genotype MHC-I diversity using Sanger and high-throughput sequencing in a species from the Charadriiformes. This bird order remains vastly underrepresented with only two known species studied that exhibit striking differences in MHC characteristics and diversity.

**Chapter III –** The lack of comparative studies between migrant, partial migrants and sedentary species (or populations), or even between species whose migratory strategies impose a differential exposure to pathogen-pressure, become essential when addressing the specific mechanistic processes behind MHC diversity (*e.g.* maximum *vs.* optimal number of alleles) and to gain a broader insight on how migrants cope with multiple parasite faunas across their flyways. We therefore compare MHC polymorphism and positive selection in two wader subspecies that differ in pathogen exposure to address their selection effects over the MHC class I repertoire.

**Chapter IV –** Using the hypothesis that animals choose their mates based on their resistance against disease, it is thought that some sexual secondary characters can reflect immune responsiveness. If this assumption holds true, we expect to see individual variation on these signal-plumage characteristics and immune investments. Black-tailed godwits have a

marked sexual dimorphism and their plumage-based pigment has an important role upon immune modulation as well as antioxidant capacities. Using breeding godwits has a study model we tested the "honest signal" hypothesis regarding innate immune system responsiveness/capacity. In other words, are males (and/or females), through their plumage characteristics, signalling to mates their ability to fight-off pathogens?

**Chapter V –** Over the past decades a combination of different immune assays is favoured in order to quantify different parts of the immune system to get a comprehensive overview of immune function. Nonetheless running several different immune assays implies multiple freeze-thaw cycles of samples, potentially promoting degradation, but the effects of these cycles over the final outcome of the immunological assays was never evaluated for birds. This study provides valuable information for applied field researchers working with wild animals and away from their laboratory facilities, and whose study conclusions largely depend on the viability of biological samples they collect.

# Black-tailed Godwits Study System

The Black-tailed godwit (Fig. 4) is a long-distance migratory wader divided into three different subspecies *limosa*, *islandica* and *melanuroides.* With a large geographical range, within the western Paleartic only the nominate (*Limosa limosa limosa*) and Icelandic (*Limosa limosa islandica*) subspecies occur (Delany et al. 2009). Godwits nest in loose colonies and like most Charadriiformes they have a fixed egg clutch of four (Delany et al. 2009; Snow and Perrins 1998). They are also a highly gregarious species, roosting and congregating in huge numbers at specific times of their annual-cycle, and this behaviour makes them particularly vulnerable to habitat loss, hunting and human disturbance (Tucker and Heath 1994).

**Fig. 4 –** Black-tailed godwit carrying on his legs an unique colour ring combination. Photo by Sara Pardal

## Nominate Godwit *Limosa limosa limosa*

The nominate (Western Europe) population estimated between 160,000-180,000 individuals, breeds mainly in the Netherlands, occupying agricultural grasslands or damp grasslands close to freshwater habitats (Delany et al. 2009). Around late June birds migrate

to their winter quarters in West Africa (*e.g*. Senegal, Guinea-Bissau, Mali) (Gill et al. 2007; Hooijmeijer et al. 2013; Delany et al. 2009) moving between freshwater areas, such as lake shores, pools, flooded grasslands and irrigated rice-fields. Outside the breeding season, they feed almost exclusively on rice, eating occasionally other small invertebrates (Lourenço and Piersma 2008). During the prenuptial migration which occurs around February-March, they perform an extended stop-over congregating in huge numbers on Iberian wetlands and overlapping with the wintering period of the Icelandic subspecies (Alves et al. 2010; Lopes et al. 2012). For godwits as well as for many other populations of Palearctic waders from the East-Atlantic flyway, Iberian wetlands are key-staging areas for both northern and southern migratory movements (Catry et al. 2011).

Despite widespread the numbers of the nominate Western Europe population had suffered large declines since 1990 in mainland Europe, especially in the Netherlands (Kuijper et al. 2006). This decline is manly owing to agricultural intensification, habitat loss and high mortality rates within youngsters (Kuijper et al. 2006). The steep decline of this subspecies was enough to trigger IUCN red list criteria leading to the recent classification of *Limosa limosa* as "Near Threatened" (IUCN 2016).

## Icelandic Godwit *Limosa limosa islandica*

The Icelandic godwit subspecies estimated in 47,000 individuals, breeds almost exclusively in the subarctic areas of Iceland, with much smaller numbers in the United Kingdom and Norway (Delany et al. 2009). Typical breeding habitats are damp grassy moorlands and bogs, but they can also be found in grasslands (Delany et al. 2009). Outside the breeding season, this subspecies migrates along the Atlantic coast, wintering mostly in Iberia, though some individuals can go further south to Morocco (Alves et al. 2010; Alves et al. 2013). At this stage, individuals preferentially occupy brackish/coastal areas such as estuaries, marshes and large intertidal mudflats, preying on estuarine benthic communities like polychaetes, bivalves and gastropods (Delany et al. 2009; Catry et al. 2012). Unlike the nominate subspecies, Icelandic godwits are growing in numbers and expanding their distribution.

Climate and agricultural changes like the availability of suitable foraging areas outside the breeding season are implicated in this trend (Gill et al. 2007).

## Migratory strategies and comparative approach

Outside the breeding season, both godwit subspecies are highly dependent on wetlands areas. Wetlands besides being extremely important habitats for several bird communities, they also congregate optimal transmission conditions for many vector-borne pathogens (Hubálek 2004, 2008), in which birds become potential hosts. However, since most arthropods are not tolerant to highly dissective conditions, high salinity areas are not suitable for most mosquito populations (and consequently mosquito-borne pathogens), and the same is true for pathogens like bacteria, which are also sensitive to salt levels (Hotchkiss 1923; Canfora et al. 2014). Therefore habitats like saline/brackish areas are likely to be poor on vector (Mendes et al. 2005; Pardal et al. 2014) and water-borne pathogens (Santos et al. 2012). At the same time, rainfall and humidity exert great influence over ectothermic arthropods like mosquitoes, but variations in temperature is probably the most important single factor regulating vector populations in temperate and tropical areas (Altizer et al. 2006). High temperature not only promotes higher infectivity rates (of vectors and hosts), but also higher parasite virulence (Reiter 2010; Altizer et al. 2006; Schmid-Hempel 2011).

Evidence for habitat and latitudinal-related differences on pathogen pressure have been reported already by several studies (Figuerola 1999; Mendes et al. 2005; Yohannes et al. 2009; Clark et al. 2015) and consequently the dichotomy on migratory strategies and habitat choice of the two subspecies of godwits lead to a differential pathogen exposure. *Icelandic godwits have a lower risk of contracting infections*, because their annual cycle is restricted to marine/coastal habitats and subarctic and temperate areas, while *nominate godwits have a higher risk of contracting infections* because they occupy freshwater/inland habitats and move between temperate and tropical areas (Fig. 5). Besides the ecological reasons, it adds the fact that both our study models are genetically similar and for instance in humans, genetics is a major contributor of vulnerability or resistance to disease (Vannberg

et al. 2011; Chapman and Hill 2012). In birds, for example Palinauskas et al. (2008) demonstrate that the severity of illness to the same strain of avian malaria infection (*Plasmodium* SGS1) was markedly different among passerine species. Differences on vulnerability to disease are therefore strongly linked with individual genetics and can be a confounding effect when comparing different species in respect to immune defence responsiveness or disease susceptibility. Once both populations share a similar genetic background (Höglund et al. 2008; Trimbos et al. 2014), differences on immune modulation and MHC polymorphism are more likely to reflect the ecological constrains of pathogen pressure. In conclusion, godwits become an excellent study system to understand how differences on pathogen pressure modulate innate and adaptive immunity, without the confounding effects of life-history strategies and genetics.

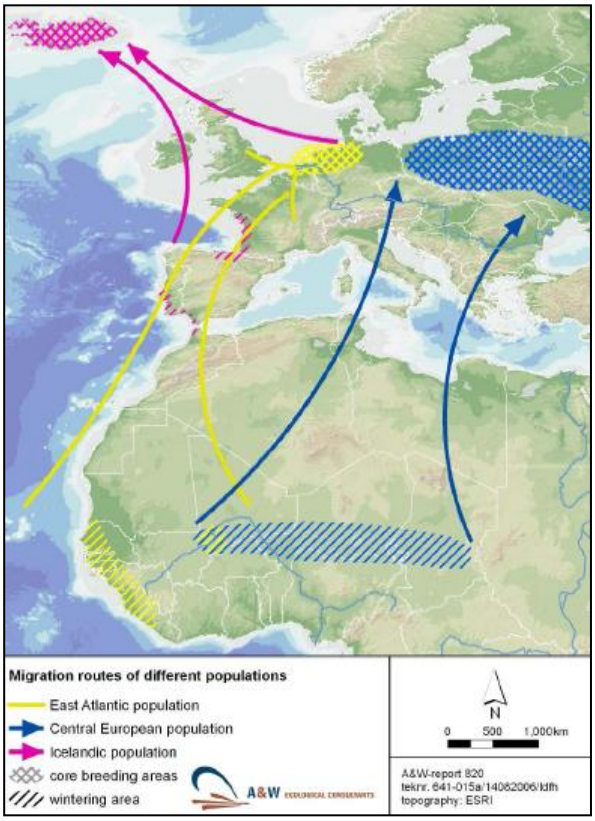



**Fig. 5 -** Migratory routes of the East Atlantic (nominate, yellow) population, the Icelandic population (pink) and the Central European population (blue) of Black-tailed godwits. Nominate and Icelandic populations are studied in the present thesis and their distribution overlap in Portuguese wetlands mostly during spring migration, but they occupy different habitats during winter. Map adapted from Kuijper *et al.* (2006).

# Chapter I

## Evidence for pathogen-mediated regulation of baseline constitutive immunity across the flyway for two shorebird subspecies


**"There is no permanent ideal of disease resistance,**
**merely the shifting sands of impermanent obsolescence."**
**Matt Ridley**

# Abstract

Immune system investment is known to vary seasonally and differs among species and life-histories, but the drivers of that variation are poorly understood. To address the effects of environmental pathogen-pressure on the immune system, we assessed the investment on baseline constitutive immunity components along the flyway of two black-tailed godwit subspecies: nominate (*Limosa limosa limosa*) and Icelandic (*L. l. islandica*). Both subspecies vary on breeding and wintering areas and habitat-use, but are genetically similar. Levels of haptoglobin, complement-mediated lysis and natural antibodies were analysed together with leukocyte profiles, and overall were not significantly different between subspecies. However, when contextualized with habitat, latitude and periods of high-energetic demands, differences on baseline immunity become apparent. During breeding, birds occupying parasite free areas like the subarctic, significantly downregulated baseline immunity features when compared to their temperate conspecifics. In winter, differences become less obvious, most likely because is a less energetic and nutritional demanding season. Along the flyway of nominate godwits, latitudinal effects on heterophil and lymphocyte levels were recorded, along with shifts on inflammatory responses. During migration, birds downregulated expensive inflammatory components, while in winter these mediators increased along with the phagocytic cell activity, an important strategy during periods of high pathogenic pressure. Habitat-related differences were also seen, with freshwater individuals upregulating phagocytic activity and inflammation mediators. We conclude that maintenance of constitutive immunity is mainly driven by seasonal factors in response to habitat-related pathogen pressure variation, and that trade-offs between immune function and competing physiological components become apparent during energetically demanding periods.

**Keywords** – immune defense, environmental pathogen-pressure, migratory flyway.

# Introduction

Animals are able to cope with environmental variations by adjusting their physiology, morphology, behaviour and annual cycles (Ricklefs and Wikelski 2002). Migration is a behavioral mechanism by which animals respond to strong seasonal variations in (particularly) food resources, by moving to areas where they are more abundant (Newton 2008). However, due to energetic and other physiological constrains, trade-offs between (and even within) competing physiological components can emerge (Hasselquist and Nilsson 2012; Sheldon and Verhulst 1996; Norris 2000; Lee 2006). One highly expensive trait, that must be regulated according to seasonal variations (Buehler et al. 2008a; Hasselquist 2007; Hegemann et al. 2012a), life-trait decisions (Norris 2000; Schmid-Hempel 2003) and genetic adjustments (Versteegh et al. 2014) is the immune system.

The immune system has evolved remarkable and intricate strategies to avoid parasite exploitation, and can be broadly divided in adaptive (specific) *vs.* innate (non-specific), and constitutive (constantly maintained) *vs.* induced (triggered by pathogen exposure) (Fox 2004). As an essential self-maintenance and survival component, each of these four axis comes with unique benefits and costs, that may lead to a wide range of immune strategies (Klasing 2004; Lee 2006). However, the trade-offs behind different immune defence investments across species and throughout annual cycles and distinct habitats are yet to be understood. Pathogen pressure is known to vary spatially (Salkeld et al. 2008; Westerdahl et al. 2014; Yohannes et al. 2009) and seasonally (reviewed in Altizer et al. 2006), and is hypothesized to be one of the reasons for the existence of two co-evolutionary migratory strategies in long-distance migrant shorebirds (Piersma 1997). Evidence for habitat and latitudinal-related differences on pathogen pressure (Figuerola 1999; Mendes et al. 2005; Yohannes et al. 2009; Clark et al. 2015), may have originated the existent dichotomy 1) high arctic shorebird breeders restricted to marine/coastal habitats, and 2) temperate breeders using freshwater/inland habitats outside the breeding season (Piersma 1997, 2003). Low pathogen risk in Arctic and marine/coastal habitats may therefore spare shorebirds from high investments in immune defence (Horrocks et al. 2011; Horrocks 2012; Sandstrom et al. 2014), allowing to redirect resources towards other traits such as thermoregulation or

parental care. This dichotomy on breeding and non-breeding habitat-use strategies by high and lower latitude breeding shorebirds is very apparent on two subspecies of black-tailed godwits (hereafter godwits) *Limosa limosa* which are extremely dependent on wetlands areas. One of the godwit subspecies, *Limosa limosa islandica* (hereafter Icelandic godwits), are subarctic breeders restricted to brackish water coastal environments, while the other subspecies, *Limosa limosa limosa* (hereafter nominate godwits) breeds on temperate areas and winters in tropical areas, mostly occupying inland freshwater habitats (Gill et al. 2007; Delany et al. 2009).

Wetlands congregate optimal transmission conditions for many vector-borne pathogens (Hubálek 2004, 2008), in which birds become potential hosts. However, since most arthropods are not tolerant to highly dissective conditions, high salinity areas are not suitable for most mosquito populations (and consequently mosquito-borne pathogens), and the same is true with pathogens like bacteria, which are also sensitive to salt levels (Hotchkiss 1923; Canfora et al. 2014). Therefore habitats like saline/brackish areas are likely to be poor on vector-borne (Mendes et al. 2005; Pardal et al. 2014) and water-borne pathogens (Santos et al. 2012). Hence, these godwit subspecies are an ideal study system in which to address pathogen pressure modulation upon the immune system, because the confounding effects of genetics, a major contributor to vulnerability or resistance to disease (*e.g.* Palinauskas et al. 2008), and relative contributions of life-history traits (*e.g.* parental investments, migratory timing, moulting period) (Ricklefs and Wikelski 2002) are very similar. Differences found in immune investments between individuals of these subspecies should therefore mainly reflect environmental constrains affecting pathogen pressure. For migrant species such as godwits that encounter a wide range of pathogens along their annual cycle (Møller and Erritzøe 1998), baseline constitutive immunity is a reliable indicator of investments on self-maintenance given that: 1) it represents the first circulating defences against infection, 2) it is non-specific and effective in controlling multiple pathogens, 3) it induces acquired immunity, 4) its responsiveness and efficiency are not influenced by prior exposure (*i.e.* it lacks immunological memory), and 5) it is a constantly maintained component of the immune system (Fox 2004; Matson et al. 2005; Buehler 2008). In order to assess how pathogen pressure and/or annual cycle periods modulate the investments on

baseline constitutive innate and adaptive immunity, we compared three parameters of baseline immunity between the two godwit subspecies, during the breeding and wintering seasons, between habitats and along their flyway. The parameters of baseline immunity investigated were: 1) circulating leukocyte (white blood cells) concentrations and relative proportions of each cell type, providing information on general health condition, stress and inflammatory processes (Davis et al. 2008; Clark et al. 2009), 2) activity levels of soluble components, such as complement (measured as lysis) and natural antibodies (NAb´s) (measured as agglutination), which facilitate phagocytosis of pathogens and dead catabolic self-particles (Ochsenbein and Zinkernagel 2000; Matson et al. 2005), and 3) haptoglobin (Hp) concentrations, an acute phase protein which levels rise to limit microbial growth, constituting a proxy for ongoing infection and inflammation (Matson et al. 2012a). Leukocyte profiles along with the complement are part of innate constitutive immunity, while Hp concerns induced innate and NAb´s belong to constitutive adaptive immunity (Lee 2006).

In terms of predicted costs (development, maintenance and use), baseline immunity variables can be broadly subdivided into three categories (Klasing 2004): high, moderate and low costs. Inflammatory mediators (*e.g.* Hp, heterophils and eosinophils), have very high metabolic requirements and indirectly lead to collateral tissue damage and anorexia, due to the large amounts of reactive oxygen and nitrogen species they produce (Lochmiller and Deerenberg 2000; Fox 2004). Activation of soluble non-specific mediators such as lysis, is also linked to inflammation and must be tightly regulated to prevent tissue damage (Rock and Kono 2008; Ashley et al. 2012), therefore the costs of their use are also hypothesized to be high (Buehler and Piersma 2008; Buehler et al. 2008a). The reliance on soluble components in blood plasma such as NAb´s, rather than phagocytosis for bacteria killing, present moderate costs in terms of energy and immunopathology (Buehler et al. 2008a) because these molecules have weak affinities and low specificities, which are important links to acquired immune defence (Ochsenbein and Zinkernagel 2000). Monocytes and lymphocytes link innate and acquired defence, as they are important mediators of antibody and cell responses, whose (energetic) costs are predicted to be low due to their high specificity (Klasing 2004). Both cells are essential for the development of long-term immunity and therefore favoured in conditions in which birds are exposed to recurrent

pathogens, or when energetic demands are high (Lee 2006). Due to the high complexity of the immune system all these mechanisms are intertwined and do not operate separately. However, depending on seasonal and physiological demands, some species do switch from rather costly non-specific inflammatory responses to more specific immunity responses (Buehler et al. 2009b), while others do not (Hegemann et al. 2012b).

We hypothesized that if baseline constitutive immunity is mainly driven by competing physiological and genetic traits, there should be no differences on those parameters between the two subspecies and/or within the same seasons and habitats. However, if habitat-driven pathogen pressure is the main modulator of baseline immunity investments, we would expect differences between individuals occupying different habitats irrespective of season and subspecies.

# Material and Methods

*Study populations and study areas*

The godwit is a long-distance migratory shorebird with a large geographical distribution, and within the western Palearctic only the nominate and Icelandic subspecies occur (Delany et al. 2009). Icelandic godwits breed in subarctic (Iceland) and nominate godwits breed in temperate latitudes (*e.g.* The Netherlands), with both subspecies selecting damp grasslands, bogs or even reclaimed land close to freshwater habitats for nesting (Delany et al. 2009). Outside the breeding season, they become extremely gregarious moving and roosting on high-density flocks (Delany et al. 2009), a disease-risk behaviour that promotes pathogen transmission between individuals (Altizer et al. 2011). During the non-breeding parts of their annual cycle, Icelandic godwits move across brackish/coastal areas such as estuaries and large intertidal mudflats, along the Atlantic coast to Iberia and Morocco for the winter (Alves et al. 2010; Alves et al. 2013). In contrast, non-breeding nominate godwits, prefer inland freshwater habitats such as lake shores, flooded grasslands and irrigated rice-fields and move further south to west African areas (*e.g.* Senegal, Guinea-Bissau) (Gill et al. 2007; Hooijmeijer et al. 2013), which are known for their higher pathogen diversity (Guernier et

al. 2004; Salkeld et al. 2008). In Iberia, nominate species can overlap during migration with wintering birds of Icelandic population (6.5%-10% *L. l. islandica*; Alves et al. 2010; Lopes et al. 2012).

Godwits were sampled along different stages of their annual cycle. During the breeding season of the Icelandic and nominate godwits (May-July 2013 and April-June 2014, respectively), incubating adults were captured with the use of nest-traps. Icelandic birds were captured in the Southern lowlands of Iceland (64°1'46.14"N, 20°59'6.04"W), while nominate individuals were captured in Friesland, northern part of The Netherlands (53°10'19.79"N, 5°46'35.60"W). During winter, autumn and spring migration, nominate godwits were captured with cannon nets or mist-nets in the Tagus estuary, Portugal (38°54'36"N, 8°56'46"W): October–February 2012-2015; Doñana, Spain (37°4'57"N, 6°8'17"W): July, September-October and January, March 2011- 2012; and Mansôa rice-fields, Guinea-Bissau (12°2'12"N, 15°37'27"W): November–December 2015. Icelandic individuals were captured on the same periods, corresponding to their wintering season, in the Tagus estuary and Doñana.

Cumulative evidences from geolocator-tagged nominate godwits, show rather disparate individual decisions regarding wintering strategy and wintering grounds (Hooijmeijer et al. 2013). Thus, nominate birds caught in Doñana were categorized as wintering birds in this study, exclusively based upon the defined “winter” period. From the 20 birds sampled during winter, only 9 of those were caught in western Africa, an absolute wintering ground for this subspecies. In total, 188 adult godwits, 62 Icelandic and 126 nominate were sampled at different stages of their annual cycle (Table 1), though not all individuals and/or seasons were analysed for every immune parameter.

**Table 1. -** Number of black-tailed godwits from each subspecies captured at different stages of the annual cycle. Nominate godwits (*L. l. limosa*): breeding (10 April – 20 June), autumn migration (21 June – 1 October), wintering (2 October – 20 November) and spring migration (21 November – 9 April). Icelandic godwits (*L. l. islandica*): breeding (May – July) and wintering (August – March).

| Life-cycle period | *L. l. islandica* | *L. l. limosa* | Total |
|---|---|---|---|
| Breeding | 29 | 47 | 76 |
| Autumn migration | 0 | 30 | 30 |
| Wintering | 33 | 20 | 53 |
| Spring migration | 0 | 29 | 29 |
| Total | 62 | 126 | 188 |

*Field sampling and blood processing*

Birds were marked with a metal ring and a color ring combination, measured (tarsus, body mass, fat and muscle scores), aged and blood sampled. Periods of blood sampling after bird capture took a median of 11 minutes during the breeding season, and 113 minutes during the remaining seasons. Blood samples of ca. 320 µl were taken from the braquial vein with a non-heparinized microhematocrit capillary tube. Two blood smears were immediately done, air-dried and stored for later processing. One part of the blood for genomic DNA extraction was kept in tubes with 96% ethanol, while the rest of the sample for baseline immunity assay was transferred to a clean eppendorf tube and kept refrigerated on ice until centrifugation for separating plasma from blood cells. Centrifugation (6.000 rpm, 10 min) was done within the 2h-12h period following sampling by using a portable field centrifuge. Serum samples were then stored at -20ºC before transportation to the laboratory and then kept at -80ºC until analysis.

*Subspecies determination*

Once the distribution of Icelandic and nominate godwits overlaps on the Iberian wintering and staging areas (Alves et al. 2010; Lopes et al. 2012), and morphological discrimination between the two is error prone, assignment of subspecies caught on the Iberian wetlands was done by a molecular assay and based on partial mtDNA variation (haplotype) (Höglund

et al. 2008). From the 103 individuals, 70 were assigned to *L. l. limosa*, while the remaining 33 individuals were *L. l. islandica.* For more detailed information, see electronic supplement.

*Baseline immunity assays*

We quantified NAb´s and complement activity of lytic enzymes, following the haemolysis-haemagglutination assay described by Matson et al. (2005) with minor modifications. Briefly, serial dilutions of serum samples from each individual were run in duplicate (12.5 µl) on microtiter plates, along with chicken plasma standards (Probiologica, Lisboa, Portugal) and incubated in a 1% rabbit RBC suspension (Probiologica, Lisboa, Portugal). Both lysis and agglutination scores reflect the last plasma dilution in the dilution series (*e.g.* rows 2 to 6) exhibiting each function. Due to serum volume limitations only 11 of the 161 samples were run only once. Scans of each individual samples were randomized, scored blindly twice and later assigned (by SP) to season, year and individual. Haemolysis was identified by the presence of free haemoglobin and lack of intact cells, while haemagglutination was identified by clumped RBC. Both lysis and agglutination scores reflect the last plasma dilution in the dilution series (*e.g.* microtiter row 6) exhibiting either of these functions. To show partial haemagglutination or haemolysis, half scores were assigned. The within-plate variation (mean ±SD) and among-plates variation, was 3.59 ±0.57 and 3.90 ±0.88 for haemolysis titres (respectively) and 9.37 ±0.63 and 9.26 ±0.39 for haemagglutination.

A commercial kit (Tridelta, Development Ltd., Maynooth, Ireland) was used to quantify the levels of circulating Hp-like proteins, following manufacturer´s instructions with minor modifications (extended standard curve to a more diluted range). Shortly, we mixed plasma and reagents in a 96-well microtiter plates and recorded absorbance at 620 nm after 5 min using a TRIAD plate reader (Triad Scientific, New Jersey, USA). Since haemolysis of RBC can affect the result of the assay, visible hemolyzed samples or those that had previously given Hp values higher than (or equal to) 0.5 mg/ml (user defined threshold level) were run again and controlled for redness following the protocol described by Matson et al. (2012a). The among-plate variation was 0.34 ±0.01 mg/ml for control 1 and 1.26 ±0.38 mg/ml for control 2. For leukocyte counts, blood smears were stained by Hemacolor Rapid

staining kit (Merck Millipore, Oeiras, Portugal), following manufacturer instructions. Microscope examination of blood smears was performed at 1000X magnification on an Olympus CX21 microscope (Olympus, Lisboa, Portugal) for leukocyte enumeration. Counts and leukocyte identification (lymphocytes, heterophils, basophils, monocytes and eosinophils) was made for the first 100 cells (Clark et al. 2009) by one observer (SP). The leukocyte density was determined by counting the numbers of WBC per 10.000 red blood cells (RBC) (equivalent to 25 vision fields at 1000X amplification) and the H/L ratio, which is the ratio between heterophils and lymphocytes (Davis et al. 2008), was also calculated after identification of 100 leukocytes.

## Statistical analysis

All response parameters were initially tested for normality using Kolmogorov-Smirnov tests and visual examination of histograms and Q-Q plots of residuals distribution. Body condition index was calculated as the standard residuals of the linear regression between body mass and tarsus length, therefore correcting for size variation between individuals. Except for body condition and haemagglutination scores, all immune parameters were transformed to obtain normality. A box-cox transformation was used for Hp concentrations, a quadratic transformation for haemolysis and, except for lymphocytes, all other leukocyte proportions were arcsine-transformed. Given that WBC, H/L ratio and lymphocyte proportions are known to suffer modifications after 30 min of handling/capture stress (Buehler et al. 2008b), we used the standard residuals of a linear regression between each variable and time elapsed between capture and sampling. As leukocyte variables were correlated (leukocyte proportions, WBC and H/L ratio), they entered a principal component analysis (PCA) using transformed data. Following the procedure described by Matson et al. (2006b), varimax normalized rotation was used to maximize variability contrasts between factor loading. The analysis identified four PCs with eigenvalues >1 that cumulatively accounted for 84.9% of the total variation of leukocyte variables: 1) PC1 had a negative correlation with lymphocyte proportions, and a positive correlation with heterophil proportions and H/L ratio, 2) PC2 had a negative correlation with eosinophil proportions, 3) PC3 had a negative correlation

with monocyte proportions, and 4) PC4 had a positive correlation with basophil proportions and total WBC. Each PC accounted respectively for 36.2%, 18.2%, 15.7%, and 14.6% of the variation (Appendix A1, available online). Individual scores for each principal component were used on subsequent analysis and were not correlated with each other (data not shown).

We used general linear and mixed models to test for the effects of subspecies, season and habitat over constitutive baseline immune parameters, controlling for the effects of body condition (covariate) (Buehler 2008). In all models only adult birds were considered and sex was treated has a fixed factor, but whenever significant it was entered as random factor in the final model. Full models contained the categorical factors subspecies (nominate - Icelandic), season, habitat (inland freshwater - coastal brackish water) and sex. We first tested if godwit subspecies generally differed in their baseline immunity parameters using season as a random factor. We then tested for the seasonal differences between the two subspecies and considering we only sampled Icelandic godwits on two points of their annual cycle (breeding and wintering), only these two seasons were directly compared between subspecies. In this comparison only African wintering nominate godwits (9 birds) were used, because only for these we were certain of the season. Comparisons were also made between seasons within the same subspecies (nominate and Icelandic separately). For the effects of habitat (nominate inland freshwater *vs.* Icelandic coastal brackish water) we used data for non-breeding birds, and entered season in the model as a random factor. For nominate godwits, data for breeding, autumn migration, wintering (Spanish and African birds included) and spring migration were used to assess the trends of baseline immunity within the entire annual cycle of godwits (except leukocyte data because it was not available for autumn migration). When the season term was significant, we used Tukey post-hoc tests to identify which seasons differed from each other. On every model, Levene tests were used to assess homogeneity of variance. To get an overview of how these baseline immunity parameters change relative to latitude (a proxy of pathogen risk), we ran Pearson correlation analysis on each immune index. All statistics were made in STATISTICA 10 (2011) and output plots of the significant differences among subspecies, seasons and habitats were created with SIGMAPLOT 12.0 (2011). Data is presented as means ± SE. Descriptive statistics for each baseline variables are presented in electronic supplement (Appendix A2).

# Results

*a) Subspecies variation in baseline constitutive immunity*

For the majority of baseline immunity variables there were no differences when comparing the two subspecies (Table 2). Only for PC4, which represents the total WBC and basophils proportion, results were significant indicating that Icelandic godwits have higher levels of both. Comparisons of baseline immunity variables between subspecies within the same season (breeding and winter), showed significant differences for 4 of the 7 variables tested, and those differences were most pronounced during breeding (Table 2). Constitutive innate variables such as haemagglutination and haemolysis, were significantly higher in nominate godwits during breeding, while in winter, there were no differences between the two subspecies (Fig. 1A and 1B). As for leukocyte variables in the breeding season, nominate godwits had significantly lower circulating lymphocytes, higher heterophils and subsequently higher H/L ratios (Fig. 2A) and lower basophil proportions and total WBC (Fig. 2D). During winter, there were no significant differences on soluble components, and only H/L ratio was higher (*i.e.* higher heterophil and lower lymphocyte levels) for nominate than for Icelandic godwits (Figure 2A).

**Table 2. –** Mixed GLM results testing for the effects of subspecies on baseline immune variables.

| Independent variables | Between subspecies | | | Between subspecies | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Winter | | | Breeding | | |
| | df | F | P* | df | F | P* | df | F | P* |
| Haptoglobin | 1,127 | 5.41 | 0.257 | 1,20 | 0.35 | 0.560 | 1,69 | 0.55 | 0.461 |
| Haemolysis | 1,122 | 0.04 | 0.880 | 1,23 | 2.47 | 0.130 | 1,69 | 23.45 | **<0.000** |
| Haemagglutination | 1,122 | 0.17 | 0.750 | 1,23 | 0.36 | 0.556 | 1,69 | 20.03 | **<0.000** |
| PC1[a] | 1,104 | 1.86 | 0.403 | 1,20 | 9.64 | **<0.006** | 1,68 | 910.34 | **<0.022** |
| PC2[b] | 1,104 | 0.42 | 0.634 | 1,20 | 10.03 | 0.166 | 1,68 | 1.11 | 0.300 |
| PC3[c] | 1,104 | 0.00 | 0.970 | 1,20 | 1.21 | 0.284 | 1,68 | 2.27 | 0.137 |
| PC4[d] | 1,104 | 233.55 | **<0.040** | 1,20 | 1.21 | 0.285 | 1,68 | 12.46 | **<0.001** |

**Note -** PC1 represents a lymphocyte, heterophil proportions and H/L ratio axis;
PC2 represents an eosinophil proportion axis;
PC3 represents a monocyte proportion axis;
PC4 represents a basophil and total WBC axis;
*Significant results (P<0.05) are in bold.

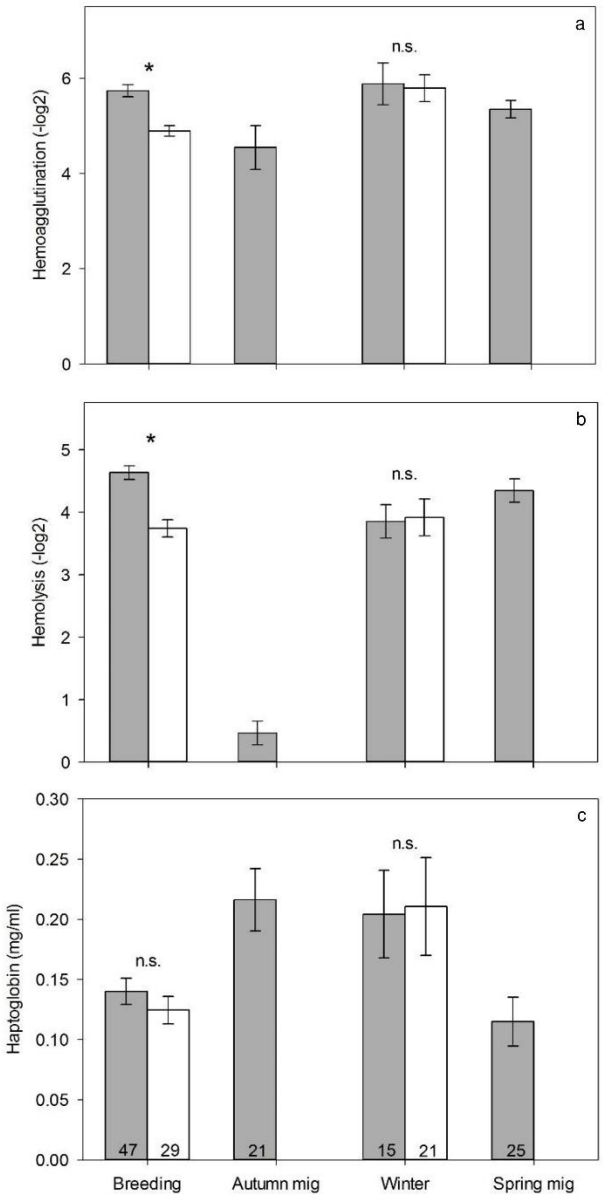



**Figure 1 -** Mean ± SE of soluble components of baseline immunity along the annual cycle for nominate (grey) and between two seasons of the Icelandic (white) godwits. (A) haemagglutination (NAb´s), (B) haemolysis, and (C) haptoglobin levels. Numbers below columns indicate sample size. Symbol * stands for statistical significant differences ($p<0.05$), and "n.s." for non-significant. All plotted variables represent significant differences between subspecies within the same season (breeding or winter), except for haptoglobin that differed significantly over the annual cycle.

*b) Seasonal variation in baseline constitutive immunity*

The between season (breeding *vs.* winter) differences in baseline constitutive immunity were more apparent in the nominate godwits than in the Icelandic subspecies (3 out of 7 variables differed significantly in nominate as opposed to 1 out of 7 in Icelandic; Table 3). Inflammation mediators, like Hp levels were significantly higher during winter for nominate godwits (Fig. 1C). The same was true for H/L ratios as circulating levels of heterophils were higher and lymphocytes levels were lower than during the breeding season (Fig. 2A). Monocytes were also less abundant in the circulating blood during winter than during breeding (Fig. 2C), and despite marginally significant the same trend was seen for eosinophils (Fig. 2B). In relation to Icelandic godwits only haemagglutination levels differed significantly between seasons, with a higher NAb´s activity during winter (Fig. 1A).

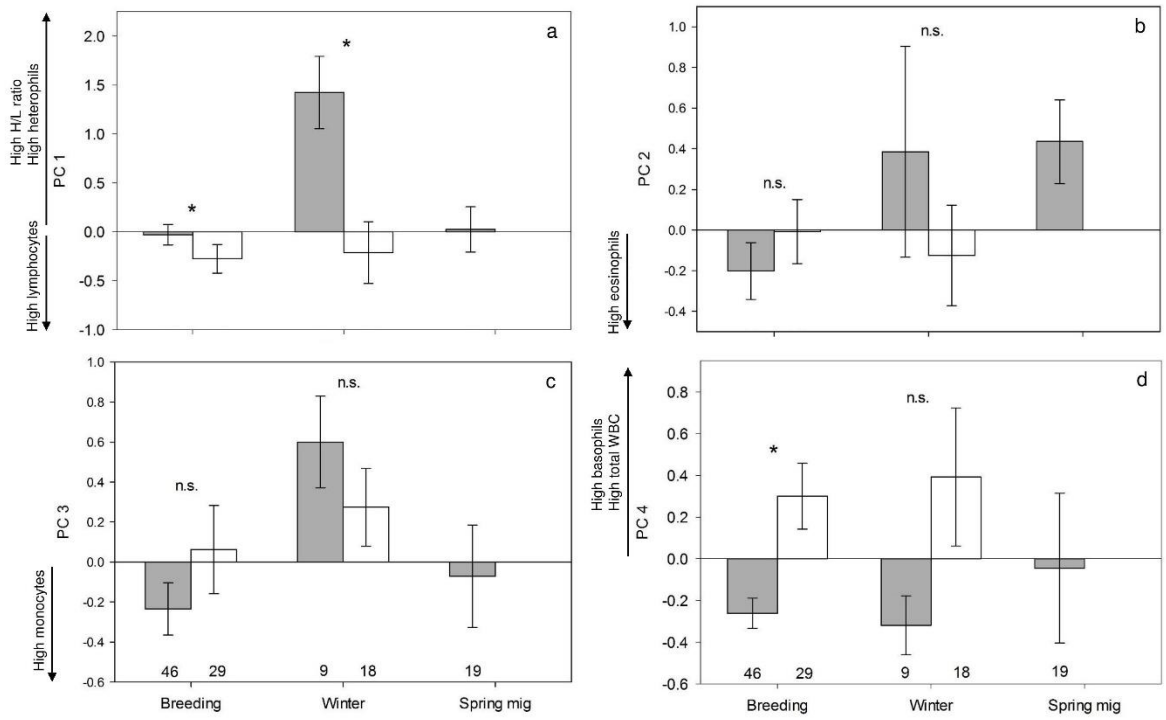



**Figure 2 –** Mean ± SE of cellular components of baseline immunity over the annual cycle for nominate (grey) and between two seasons for the Icelandic (white) godwits. Numbers below columns indicate sample size. Symbol * stands for significant differences ($p<0.05$), while "n.s." for non-significant. Except for PC2 and PC3 whose values where significantly different along the annual cycle, all plotted variables represent significant differences between subspecies within the same season (breeding and

winter). Plots from (a) to (c) represent the leukocyte variables grouping axes after PCA analysis. PC1 (a) was negatively correlated with lymphocyte proportions and positively correlated with heterophil proportion and H/L ratio. PC2 (b) was negatively correlated with eosinophil proportion and PC3 (c) was negatively correlated with monocyte proportion. PC4 (d) was positively correlated with basophil proportions and total WBC.

*c) Annual cycle variation of baseline immunity of nominate godwits*

All baseline immunity variables, except three (haemagglutination, PC1 and PC4), varied significantly along the annual cycle of nominate godwits (Table 3). Hp levels were lowest during spring migration and highest during autumn migration and winter (Fig. 1C). During breeding and spring migration, haemolysis activity was significantly higher, declining substantially in autumn migration and increasing during winter (Fig. 1B). Despite marginally significant, levels of haemagglutination during breeding reached their highest, differing significantly from the lowest levels of autumn migration, while for the rest of the seasons, changes were not significant (Fig. 1A). The trends seen on H/L ratio and circulating heterophils and lymphocytes (Fig. 2A) along the annual cycle, were strongly driven by sex effects, with males having higher levels of H/L ratio, circulating heterophils and lower levels of lymphocytes than females (data not shown) across all seasons. Eosinophil levels were highest during breeding and similarly lowest during winter and spring migration (Fig. 2B). As for monocyte levels they were higher during breeding, decreasing substantially during the winter season, and rising again during spring migration (Fig. 2C). As for total WBC and basophils proportion, and despite the no significant differences between seasons, both were lowest in winter and breeding, and increased a bit during spring migration (Fig. 2D).

**Table 3. –** Mixed GLM results testing for the effects of season (between breeding and wintering for each subspecies and across the annual cycle for nominate subspecies) and habitat on baseline immune variables.

| Independent variables | Between seasons | | | | | | Nominate annual cycle stages | | | Habitat | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Nominate | | | Icelandic | | | | | | | | |
| | df | F | P* | df | F | P* | df | F | P* | df | F | P* |
| Haptoglobin | 1,52 | 4.55 | **<0.038** | 1,40 | 1.49 | 0.230 | 3,86 | 4.41 | **<0.006** | 1,57 | 0.84 | 0.362 |
| Hemolysis | 1,49 | 14.27 | 0.164 | 1,43 | 1.58 | 0.216 | 3,78 | 60.87 | **<0.012** | 1,52 | 2.72 | 0.105 |
| Hemagglutination | 1,49 | 0.46 | 0.503 | 1,43 | 13.47 | **<0.001** | 3,78 | 2.59 | 0.058 | 1,52 | 0.29 | 0.594 |
| PC1[a] | 1,48 | 829.36 | **<0.012** | 1,40 | 0.02 | 0.879 | 2,63 | 367.04 | 0.830 | 1,35 | 10.39 | **<0.003** |
| PC2[b] | 1,48 | 3.70 | 0.060 | 1,40 | 2.01 | 0.164 | 2,63 | 3.71 | **<0.030** | 1,35 | 4.53 | **<0.040** |
| PC3[c] | 1,48 | 8.90 | **<0.004** | 1,40 | 0.78 | 0.381 | 2,63 | 4.57 | **<0.014** | 1,35 | 1.02 | 0.320 |
| PC4[d] | 1,48 | 0.17 | 0.680 | 1,40 | 0.00 | 0.966 | 2,63 | 1.14 | 0.326 | 1,35 | 1.07 | 0.309 |

**Note -** PC1 represents a lymphocyte, heterophil proportions and H/L ratio axis;
PC2 represents an eosinophil proportion axis;
PC3 represents a monocyte proportion axis;
PC4 represents a basophil and total WBC axis;
*Significant results (P<0.05) are in bold and marginally non-significant results are underlined.

*d) Habitat differences*

When comparing baseline immunity variables between individuals occupying different habitats outside the breeding season, significant differences were only seen at the cellular level (Table 3). Nominate godwits from inland freshwater habitats had higher levels of H/L ratios and proportions of circulating heterophils, and lower levels of lymphocytes and eosinophil than Icelandic godwits (Appendix A3, available online). No significant differences were seen at soluble components. Person's correlations of baseline values across a latitudinal gradient, indicate significant trends for the proportion of circulating heterophils and lymphocytes and H/L ratios (Appendix A4, available online): heterophils and H/L ratios increased, and lymphocytes decrease with decreasing latitude, respectively (Fig. 3A). The levels of Hp were also marginally significant, increasing with decreasing latitude (Fig. 3B).

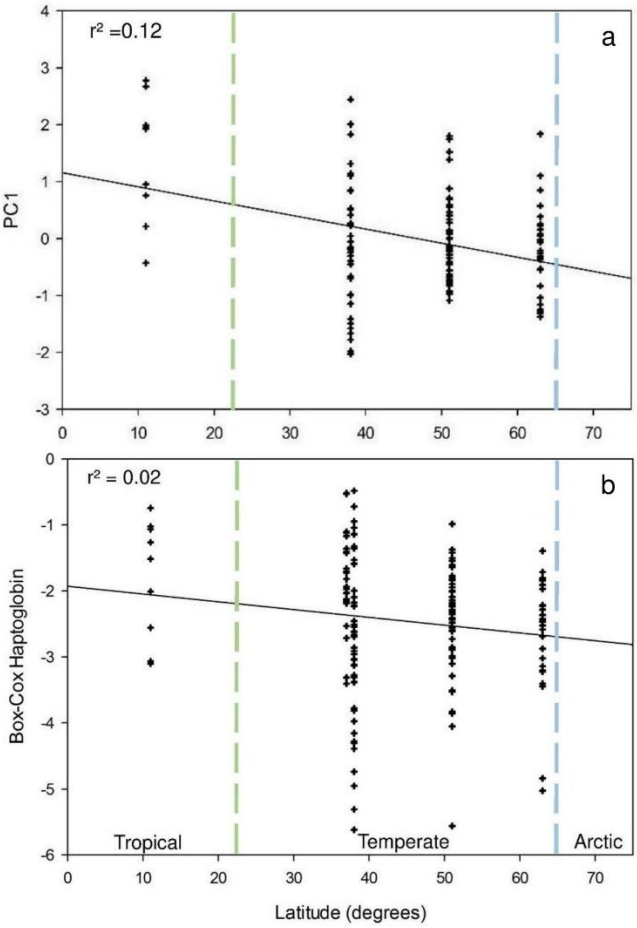



**Figure 3** - (a) PC1 (reflecting H/L ratio and thus opposing trends of heterophils and lymphocytes), and (b) haptoglobin levels as function of latitude along the flyway of Black-tailed godwits. PC1 is positively correlated with proportion of heterophils and H/L ratios and negatively correlated with lymphocyte proportions. Green and blue dashed lines represent the Tropic of Cancer and Artic Circle, respectively.

# Discussion

Results showed that maintenance of constitutive immunity is mainly driven by seasonal factors in response to habitat driven variation in pathogen pressure, and that trade-offs between competing physiological components are more evident during periods of physiological strain, such as the breeding season.

*Phenotypic flexibility between the two godwit subspecies*

Nominate and Icelandic godwits did not differ on most of the baseline immune parameters when season was statistically accounted for; the only significant differences were total WBC and basophils proportions. Despite no information on the "normal" WBC values for godwits, higher values for the Icelandic subspecies could be an indication of imposed higher osmoregulatory stress of occupying saline habitats (Gutiérrez et al. 2013). Overall these results are not surprising given that the two godwit subspecies are genetically very similar (Höglund et al. 2008).

*Post-breeding baseline immunity variation: physiological and seasonal effects*

Studies of immune function during the non-breeding season present contradictory results, with some predicting that it should be more robust (Nelson and Demas 1996; Martin et al. 2008), as birds need to enhance it in the advent of the harsh winter condition and/or immunosuppressive effects of reduced food availability, while others found no support for this hypothesis (Owen-Ashley and Wingfield 2006; Martin II et al. 2004). For migratory species, a higher level of immune system flexibility could be expected, as individuals are likely to encounter novel (or previous) pathogens at stop-over and wintering locations (Hasselquist 2007; Versteegh et al. 2014; Westerdahl et al. 2014) this is definitely the case for nominate godwits, because not only is there higher variation in constitutive immune investments between breeding and wintering periods when compared to Icelandic godwits, but also higher variation between the migratory periods. Godwit annual cycle immune patterns are not only different from those found in passerines (Hegemann et al. 2012a; Versteegh et al. 2014), but also for closely related species, such as red knots (*Calidris canutus*) (Buehler et al. 2008a) and even between godwit subspecies. This is presumably an indication of some phenotypic flexibility over baseline immune components, besides a response to habitat stressors and different seasonal demands. During winter, nominate godwits seem to rely more on inflammatory mediators, such as Hp and heterophils, while the lower profile levels seen for lymphocyte, eosinophil and monocyte along with higher H/L ratios are indicators of physiological stress and/or infection (Davis et al. 2008). The upregulation of inflammatory mediators and phagocytic cell activity by nominate godwits can provide a quick protection against novel and fast replicating pathogens, and may be an

important strategy during periods of higher pathogen pressure in the tropical wintering areas. At the same time, the involved costs of such immune strategy seem to be affordable in a period when birds are free from the thermoregulatory costs, immunosuppression effects of breeding hormones and nutritional effects (see Buehler et al. 2009b, 2010). It should be noted that godwits were caught well after arrival to wintering areas (Kleijn et al. 2010) and had an advance stage of fat deposition (mean fat score = 4.1; scale 0-8, Kaiser 1993). Within shorebirds and outside the breeding season, a higher investment on cellular-mediated immunity (though sex-biased) was found in ruffs (*Philomachus pugnax*) (Lozano and Lank 2003), which also prefer freshwater habitats. Buehler et al. (2009a) found an overall immune suppression of baseline constitutive values for tropical wintering red knots, a more coastal species. The rather opposite trends might be due to differential immune function activation approach (induced cell-mediated *vs.* constitutive innate), and trade-offs within the immune system, or even habitat and behavioral-related differences between shorebird species (Mendes et al. 2006a). By comparison, the baseline immunity of Icelandic godwits showed little variation from breeding to winter, with only haemagglutination levels rising significantly during winter. Indeed, from all the immune parameters taken into account, NAb´s levels seemed to be the least plastic over the year suggesting an independent regulation, which is consistent with other studies (Mendes et al. 2006a; Buehler et al. 2008a; Versteegh et al. 2014). The apparently less costly immune strategy of Icelandic godwits seems to be enough for the earlier recognition and clearance of pathogens when on temperate areas, such as Iberia.

Studies during migration indicate shifts (up or down regulation) in immune investments during this period (Buehler et al. 2008c, 2010; Hegemann et al. 2012a; Versteegh et al. 2014; Eikenaar and Hegemann 2016). Suppression of innate immunity was found in a recent study by Eikenaar and Hegemann (2016) with short-distance migratory blackbirds (*Turdus merula*), so it seems logical that reductions are more substantial when it comes to long-distance migrants such as godwits. The two migratory periods of nominate godwits are energetically demanding (Alves et al. 2013), and in both periods godwits downregulated expensive components of the immune system such as haemolysis, heterophils proportions and Hp, but the degree of change was more pronounced during autumn migration, a period in which birds perform body and primary moult at the same time

(Pienkowski et al. 1976). As nominate godwits are coming from their breeding areas, it seems that the overlapping effects of migration, extensive moult and possibly lagging immunosuppressive effects of breeding hormones may reduce the investment on immune defense. Nonetheless, immune investments could also be a reflection of the lower parasite pressure (*e.g.* vector-borne) that birds experience during stop-over periods at freshwater temperate areas (Pardal et al. 2014), or the result of food shortage on rice-fields (Hooijmeijer et al. 2013; Kuijper et al. 2006) that directly can affect activation of inflammatory responses (Buehler et al. 2009b, 2010). Our results also suggest that during autumn migration nominate godwits do not enhance their immune system in anticipation for the higher parasitic pressure on tropical wintering areas, similarly to the results obtained for red knots by Buehler et al. (2009a).

*Pathogen-mediated stressors of baseline immunity: latitudinal and habitat effects*

The capacity of geographic and habitat differences to modulate the immune system, can be seen during the breeding and wintering season of both godwit subspecies. Arctic and subarctic areas are parasite-poor habitats, and a downregulation of the immune function could be associated with the strategic use of those areas for breeding, sparing Icelandic godwits from the energetic costs of immune enhancement and allowing a direct investment into parental care. This may explain the downregulation of moderate to costly immune components of constitutive innate and adaptive nonspecific defense, such as haemolysis, haemagglutination and heterophils proportions (Buehler and Piersma 2008). The higher H/L ratio for nominate godwits, and considering that both subspecies are under the same energetic constrains (breeding), suggests that nominate birds undergo higher physiological stress/inflammatory processes during the breeding season. Higher workloads, predator pressure and melatonin levels also contribute to downregulate immune function (Navarro et al. 2004; Hasselquist 2007; Hegemann et al. 2013a). However, we have no reasons to believe that these factors may have influenced the final trends. Both populations had similar predation rates (22-32% for nominate breeding season, Jos Hooijmeijer, unpublished data; 34% for Icelandic godwits, JAA *pers. obs.*), similar clutch sizes (mean of 4 eggs) and were caught when chicks were hatching. As for melatonin, though we cannot exclude that this

circannual hormone may have played a role on immune suppression in Icelandic godwits, Buehler et al. (2009c) found no evidence of melatonin modulation on red knots, a close relative of godwits and other arctic breeders. The observed baseline immune patterns between subarctic and temperate breeders, were also found on barnacle goose (*Branta leucopsis*) (Sandstrom et al. 2014), and are in line with previous studies on habitat-related differences in immune activation (Buehler 2008, Buehler et al. 2008c, 2009a, Horrocks et al. 2011, 2012; Horrocks and Matson 2012). To the best of our knowledge this is the first study with free-living animals, which eliminates the confounding effects of genetic (*i.e.* between subspecies) and life-history trait differences, showing a clear effect of pathogen pressure upon modulation of the immune function.

Outside the breeding season, the habitats occupied by the two subspecies differ quite substantially in terms of pathogens, and theoretically we would expect an upregulation of baseline immunity (Piersma 1997; Lindström et al. 2004; Mendes et al. 2005). However, significant differences for nominate godwits when in tropical areas (winter) and habitats, were only found at the cellular level. Upregulated immunity features in tropical and freshwater areas are associated with non-specific and immediate defense, and may reflect ongoing infectious processes/higher stress (Davis et al. 2008) and/or an upregulation of pathogen killing through phagocytic activity. This immune strategy can be beneficial and its higher costs outweighed, when birds encounter bacteria, yeast or novel pathogens in tropical and/or freshwater areas. Regarding tropical wintering areas, larger variation surrounding Hp, haemolysis and haemagglutination levels (data not shown), could be a reflection of the lower sample size (N=9 African birds), or may reflect latent infections during this season. As a proxy for disease risk, Pearson correlations did show increases in inflammatory and physiologic stress indices (*i.e.* haptoglobin) with decreasing latitude. Despite the marginally significant and weak results for Hp, it seems biologically relevant that levels of an acute phase protein with an important inflammatory role increase when pathogen exposure is higher. A similar trend in Hp was recorded by Horrocks et al. (2012) for several lark species, with birds occupying more humid and wet environments having higher levels of Hp. Another explanation for the lack of significant differences in some immune measures between the two subspecies during winter could be related to the fact that energetic demands are lower and therefore differences in immune investments become less obvious. Iberian sites for

staging and wintering shorebirds proved to be a good quality refuelling area during spring migration (Martins et al. 2013) and for wintering Icelandic godwits (Alves et al. 2013). As for the nominate godwits caught in Guinea-Bissau, they were also in a good body condition (*pers. obs.*). For the immunity differences found at the habitat level, previous studies by Mendes et al. (2006) when comparing the acquired and innate immune response of five shorebird species with differential habitat use, found no broad pattern of immunity between habitats. Regarding soluble baseline components like NAb´s and complement activity (measured has haemagglutination and haemolysis), our results of no difference between habitats are in line with those found by Mendes et al. (2006). However, at the cellular level results followed the same trends as Buehler et al. (2008a) for captive and free-living red knots. An alternative explanation could be a downregulation of NAb´s and complement activity in Icelandic godwits, caused by adrenocortical hormone regulation of salt glands. Recent work with dunlins (*Calidris alpina*) indicate that osmotically challenging conditions may suppress some immune functions (Gutiérrez et al. 2013) and though we did not test cell-mediated immunity, our results may indicate either an upregulation of the non-specific immunity by freshwater individuals, or a less flexibility of coastal birds to upregulate their immune system. In conclusion, we emphasize the need to further study the relationship between the role of osmoregulatory hormones and immunity of birds occupying freshwater and brackish areas.

# Appendix

**Appendix A1** Principal component loadings after varimax rotation. Bold values represent the leukocyte variables that were significantly correlated with each axis.

| Variable | PC1 | PC2 | PC3 | PC4 |
|---|---|---|---|---|
| Lymphocyte proportion | **-0.891** | 0.374 | 0.151 | 0.063 |
| Heterophil proportion | **0.899** | 0.308 | 0.115 | -0.010 |
| Eosinophil proportion | -0.021 | **-0.988** | 0.051 | -0.041 |
| Basophil proportion | 0.037 | 0.090 | -0.264 | **0.818** |
| Monocyte proportion | -0.044 | 0.028 | **-0.916** | 0.050 |
| WBC | -0.192 | -0.052 | 0.398 | **0.657** |
| H/L ratio | **0.922** | 0.025 | 0.017 | -0.082 |
| Variance (%) per component | 36.2 | 18.2 | 15.7 | 14.6 |
| Cumulative variance | 36.2 | 54.5 | 70.2 | 84.9 |

**Appendix A2 –** Descriptive statistics of baseline immune variables of nominate and Icelandic godwits across the annual cycle. Prop. stands for proportion; Std. stands for standard residuals.

| | Breeding | | | | | Autumn migration | | | | | Winter | | | | | Spring migration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nominate godwits | N | Mean | Min | Max | SE | N | Mean | Min | Max | SE | N | Mean | Min | Max | SE | N | Mean | Min | Max | SE |
| Body condition (Std. residuals) | 45 | 0.321 | -0.973 | 1.779 | 0.109 | 30 | -0.358 | -1.324 | 1.341 | 0.137 | 20 | 0.095 | -1.007 | 2.326 | 0.185 | 29 | -0.340 | -2.947 | 2.075 | 0.297 |
| Haptoglobin (mg/ml) | 47 | 0.140 | 0.015 | 0.395 | 0.011 | 21 | 0.216 | 0.059 | 0.605 | 0.026 | 15 | 0.204 | 0.060 | 0.491 | 0.036 | 25 | 0.115 | 0.015 | 0.409 | 0.020 |
| Hemolysis ($-\log_2$) | 47 | 4.633 | 3.250 | 6.000 | 0.110 | 14 | 0.464 | 0.000 | 1.750 | 0.189 | 12 | 3.854 | 2.500 | 5.250 | 0.267 | 26 | 4.346 | 2.000 | 6.000 | 0.187 |
| Hemagglutination ($-\log_2$) | 47 | 5.731 | 4.000 | 8.000 | 0.127 | 14 | 4.542 | 2.000 | 8.625 | 0.459 | 12 | 5.878 | 4.125 | 9.167 | 0.441 | 26 | 5.345 | 4.000 | 7.250 | 0.184 |
| Lymphocytes (prop) | 46 | 0.539 | 0.239 | 0.800 | 0.020 | - | - | - | - | - | 9 | 0.281 | 0.146 | 0.407 | 0.030 | 19 | 0.479 | 0.250 | 0.800 | 0.035 |
| Heterophils (prop) | 46 | 0.271 | 0.064 | 0.519 | 0.017 | - | - | - | - | - | 9 | 0.600 | 0.136 | 0.778 | 0.069 | 19 | 0.393 | 0.100 | 0.608 | 0.032 |
| Eosinophils (prop) | 46 | 0.151 | 0.000 | 0.432 | 0.014 | - | - | - | - | - | 9 | 0.111 | 0.000 | 0.500 | 0.053 | 19 | 0.094 | 0.000 | 0.315 | 0.019 |
| Basophils (prop) | 46 | 0.000 | 0.000 | 0.017 | 0.000 | - | - | - | - | - | 9 | 0.000 | 0.000 | 0.000 | 0.000 | 19 | 0.003 | 0.000 | 0.048 | 0.002 |
| Monocytes (prop) | 46 | 0.038 | 0.000 | 0.139 | 0.005 | - | - | - | - | - | 9 | 0.008 | 0.000 | 0.071 | 0.008 | 19 | 0.031 | 0.000 | 0.100 | 0.009 |
| Total WBC | 46 | 48 | 21 | 84 | 2.386 | - | - | - | - | - | 9 | 31 | 18 | 52 | 3.962 | 19 | 32 | 4 | 84 | 4.399 |
| H/L ratio | 46 | 0.635 | 0.088 | 2.200 | 0.072 | - | - | - | - | - | 9 | 2.525 | 0.375 | 5.143 | 0.487 | 19 | 1.108 | 0.167 | 4.150 | 0.191 |
| Icelandic godwits | | | | | | | | | | | | | | | | | | | | |
| Body condition (Std. residuals) | 29 | 0.152 | -0.962 | 1.685 | 0.144 | - | - | - | - | - | 32 | -0.030 | -2.304 | 1.870 | 0.187 | - | - | - | - | - |
| Haptoglobin (mg/ml) | 29 | 0.124 | 0.021 | 0.278 | 0.011 | - | - | - | - | - | 21 | 0.211 | 0.025 | 0.627 | 0.041 | - | - | - | - | - |
| Hemolysis ($-\log_2$) | 29 | 3.741 | 2.000 | 5.000 | 0.137 | - | - | - | - | - | 24 | 3.917 | 0.000 | 6.000 | 0.293 | - | - | - | - | - |
| Hemagglutination ($-\log_2$) | 29 | 4.888 | 3.875 | 6.125 | 0.110 | - | - | - | - | - | 24 | 5.786 | 3.583 | 9.375 | 0.280 | - | - | - | - | - |
| Lymphocytes (prop) | 29 | 0.586 | 0.246 | 0.875 | 0.031 | - | - | - | - | - | 18 | 0.460 | 0.136 | 0.823 | 0.042 | - | - | - | - | - |
| Heterophils (prop) | 29 | 0.246 | 0.021 | 0.624 | 0.024 | - | - | - | - | - | 18 | 0.353 | 0.056 | 0.731 | 0.049 | - | - | - | - | - |
| Eosinophils (prop) | 29 | 0.127 | 0.000 | 0.327 | 0.016 | - | - | - | - | - | 18 | 0.159 | 0.000 | 0.364 | 0.023 | - | - | - | - | - |
| Basophils (prop) | 29 | 0.001 | 0.000 | 0.010 | 0.000 | - | - | - | - | - | 18 | 0.003 | 0.000 | 0.030 | 0.002 | - | - | - | - | - |
| Monocytes (prop) | 29 | 0.040 | 0.000 | 0.156 | 0.008 | - | - | - | - | - | 18 | 0.025 | 0.000 | 0.143 | 0.008 | - | - | - | - | - |
| Total WBC | 29 | 70 | 7 | 143 | 5.808 | - | - | - | - | - | 18 | 45 | 16 | 85 | 4.359 | - | - | - | - | - |
| H/L ratio | 29 | 0.586 | 0.027 | 2.536 | 0.106 | - | - | - | - | - | 18 | 1.239 | 0.083 | 3.667 | 0.270 | - | - | - | - | - |

# Chapter II

## Characterization of MHC class I in a long distance migratory wader, the Icelandic black-tailed godwit

**"Nothing in biology makes sense except in the light of evolution."**

**Theodosius Dobzhansky**

# Abstract

The Major Histocompatibility Complex (MHC) is a key protein in antigen presentation and pathogen elimination. MHC class I (MHC-I) genes have gained a large interest among researchers in ecology and evolution and have been partly characterized in wide range of bird species. So far, the main focus has been on species within the bird orders Galliformes and Passeriformes, while Charadriiformes remains vastly underrepresented with only two species studied. These two species exhibit striking differences in MHC-I characteristics and diversity. We therefore set-out to study a third species within Charadriiformes, the Icelandic subspecies of black-tailed godwits (*Limosa limosa islandica*). This subspecies is normally confined to parasite-poor environments and we hence expected a rather low MHC diversity. MHC-I was thoroughly characterized using Sanger sequencing and then 84 individuals were genotyped with high-throughput sequencing (MiSeq). We verified 47 alleles in open reading frame with classical MHC-I characteristics in the godwits and each individual had two to seven putatively classical MHC alleles. However, in contrast to previous MHC-I data within Charadriiformes we did not find any evidence of non-classical genes. Godwits had an MHC-I diversity and polymorphism falling in between the previous estimates within Charadriiformes. Interestingly, godwits had fewer sites subject to positive selection and one possible explanation could be a low exposure to pathogens throughout their annual migrations.

**Keywords:** Major Histocompatibility Complex, MHC class I, *Limosa limosa islandica*, Charadriiformes.

# Introduction

The major histocompatibility complex (MHC) is an important contributor of the adaptive immunity (Neefjes et al. 2011). MHC genes are highly polymorphic and this characteristic along with the function of MHC proteins in immunity make MHC particularly relevant for studies of molecular evolution. The molecular footprints in the MHC genes, as well as MHC allele frequencies within populations, are expected to reflect evolutionary and adaptive processes both within and among populations (Hess and Edwards 2002; Sommer 2005). MHC genes are found in all vertebrates and exist in a multigene family, where class I (MHC-I) and class II (MHC-II) are the two main groups having high genetic polymorphism and known function in antigen presentation. Though MHC-I and MHC-II proteins have a similar overall structure they operate in slightly different ways by presenting peptides (antigens) from the intracellular (representing *e.g.* viruses) and extracellular (representing *e.g.* nematodes, cestodes and many bacteria,) environment, respectively (Neefjes et al. 2011). MHC-I proteins are found on all nucleated cells and if the presented peptide is the product of foreign or transformed antigens, it is recognized as foreign by T-cells which will kill the infected/transformed cell (Neefjes et al. 2011). The antigen presentation by MHC proteins is the key initial step for triggering an adaptive immune response and the residues in the peptide binding region (PBR) of the MHC proteins are therefore particularly interesting when studying evolutionary processes, such as host-pathogen interactions, at the molecular level.

The first studies of MHC characterization in avian species were done already in the 1950's with the domestic chicken (*Gallus gallus domesticus*) as the model species. Domestic chicken has two classical MHC-I genes and birds in general were for long thought to have few of MHC genes (Kaufman et al. 1999). Subsequent studies on gene expression of additional Galloanserae species, such as the Japanese quail (*Coturnix japonica*) and the mallard (*Anas platyrhynchos*), with two and one highly expressed gene copies, respectively, further supported the finding that birds in general have or express a limited number of MHC genes (Shiina et al. 1999; Moon et al. 2005). However, partial characterization of MHC-I in additional bird orders, Passeriformes (songbirds) in particular, have proven the existence of

large numbers of MHC-I gene copies at the genomic level, though the number of expressed genes is yet to be thoroughly investigated (Westerdahl et al. 1999; Sepil et al. 2012; Karlsson and Westerdahl 2013; O'Connor et al. 2016, but see Drews et al. *submitted*).

When high-throughput sequencing (HTS) became feasible for large scale MHC genotyping in non-model organisms it boosted the number of bird species for which there are good estimates of MHC allelic diversity (Zagalska-Neubauer et al. 2010; Sepil et al. 2012; Karlsson and Westerdahl 2013). MHC diversity has primarily been studied within species on a micro-evolutionary scale but more recently also on the macro-evolutionary level across species (O'Connor et al. 2016). Nonetheless these HTS studies on MHC diversity have so far mostly been restricted to passerines while there is lack of knowledge in other bird orders, hence limiting the potential for broader comparative analyses. We therefore set out to firstly partly characterize MHC-I and secondly genotype MHC-I diversity in a species from the bird order Charadriiformes.

Species within Charadriiformes are particularly interesting from a host-pathogen perspective since this group includes long-distance migrants with a wide range of migratory strategies resulting in striking differences regarding pathogen exposure (Piersma 1997, 2003; Delany et al. 2009; Clark et al. 2015). MHC-I has previously only been characterized in two distantly related Charadriiformes species, the red knot (*Calidris canutus;* hereafter knot) and the red-billed gull (*Larus scopulinus;* hereafter gull). These species differ in both genetic structure and in overall genetic diversity (Cloutier et al. 2011; Buehler et al. 2013). Our study species the black-tailed godwits (*Limosa (l.) limosa*), specifically the Icelandic subspecies (*L. l. islandica*; hereafter godwits) is restricted to relative parasite-free areas and we therefore expect a rather low MHC-I diversity. In the present study we (1) partly characterize MHC-I in godwits using Sanger sequencing on long transcripts, (2) genotype MHC-I diversity in a breeding population of godwits using HTS (Illumina MiSeq), (3) investigate genetic polymorphism and evidence of positive selection in godwit MHC-I and (4) finally, compare the MHC-I genetic organisation and diversity in three species from the order Charadriiformes, godwits, knots and gulls.

# Material and methods

*Study subspecies and fieldwork*

*The godwit is a migratory shorebird distributed over large discontinuous* breeding and wintering ranges and within the western Palearctic only the nominate (*L. l. limosa*) and Icelandic subspecies occur (Delany et al. 2009). The Icelandic subspecies breed predominantly on Iceland and outside the breeding season the birds move along the Atlantic coast from Britain to Morocco, often in brackish habitats such as sheltered estuaries, lagoons and large intertidal mudflats (Gill et al. 2007; Delany et al. 2009; Alves et al. 2010; Alves et al. 2013). We captured incubating adults during the breeding season (May to July of 2011, 2012 and 2013) on the southwestern part of Iceland (64°1'46.14"N, 20°59'6.04"W) with the use of nest-traps. Captured birds were blood sampled from the brachial vein (ca. 90 µl) ringed and released unharmed. Blood for genomic DNA (gDNA) was kept in 96% ethanol, while for RNA blood was transferred to a NUNC tube filled with 500 µl RNAlater (Thermo Fisher Scientific/Ambion, Waltham, USA). DNA and RNA samples were kept at -20ºC, during fieldwork and then stored at -80ºC until laboratory analysis. In total, this study is based on 84 samples of genomic DNA collected in 2011-2013 and one RNA sample collected in 2013.

*Extraction, gDNA and cDNA preparation*

Total genomic DNA was extracted by an adapted ammonium acetate protocol (Richardson et al. 2001). RNA extraction and purification was done using a combination of the TRIzol LS manufacturer protocol (Life Technologies, Carlsbad, CA, USA) and the RNeasy Mini kit manufacturer protocol (QIAGEN, Hilden, Germany). To reverse transcribe the RNA (mRNA) to complementary cDNA we used RETROscript Kit according to the manufacturer's instructions (Thermo Fisher Scientific/Ambion, Waltham, USA). See online resource Material and Methods for detailed information.

*Long MHC-I transcripts: Primer design, molecular cloning and Sanger sequencing*

Primer pairs for amplification of MHC-I in the godwits were either newly designed or from a previous study by Strandh et al. (2011) (Fig. 1; online resource Table S1). A first set of new primers that amplified approximately 736 bp, covering partial exon 2 to partial exon 4, was designed based on an alignments consisting of MHC class I sequences from domestic chicken (NM001030675.1; HQ141386.1), Japanese quail (AB005528.1), mallard duck (AB115239.1), great reed warbler (*Acrocephalus arundinaceus*, AJ005503.1), red-billed gull (HM008714.1; HM008715.1; HM008713.1; HM008716.1), blue petrel (*Halobaena caerulea*, JF276881.1; JF276884.1), red knot (KC205119.1; KC205120.1, KC205121.1; KC205116.1; KC205117.1; KC205107.1; KC205113.1) and Florida sandhill crane (*Grus canadensis pratensis*, AF033106.1) downloaded from GenBank database (Benson et al. 2013). By adding successful godwit cDNA and gDNA sequences (*i.e.* short exon 2 sequences and long exon 3 to exon 4 sequences) to the previous alignment a new set of primers could be designed that amplified the variable exon 2 and 3 regions (holding the PBR, covering the major part of both exons 519 bp out of 540 bp full length). All MHC fragments were amplified using 10 ng of cDNA or 25 ng gDNA as template with standard PCR protocols, AmpliTaq polymerase kits (Applied biosystems, New Jersey, US) and annealing temperatures specified in online resource Table S1. The PCR products were visualized on 2% agarose gels by electrophoresis and cloned using TOPO TA Cloning Kit with pCR 2.1 TOPO vector and One Shot Chemically competent cells, following manufacturer's instructions (Invitrogen, Paisley, UK). Selected plasmid clones were lysed in 150 μl of dd$H_2O$ at 95ºC for 3 min. Afterwards PCR amplification was done using 1-2 μl of lysed plasmids as template, and the forward primer M13F and reverse primer M13R (Invitrogen, Paisley, UK). Inserts of the correct length were identified on 2% agarose gels and the PCR products were precipitated with ammonium acetate (8.0 M) and ethanol. Finally, the purified product was used as template in a BigDye terminator Sanger sequencing reaction using BigDye terminator kit v.3.1 (Applied biosystems, New Jersey, US). Sequences were obtained from an ABI PRISM 3130 genetic analyzer (Applied biosystems, New Jersey, US) and the results were edited in BioEdit 7.2.5 Sequence Alignment Editor (Hall 1999) and Geneious version 8.1.7 (http://www.geneious.com; Kearse et al.

2012). Altogether 85 cDNA and 15 gDNA high quality sequences were retrieved using nine different primers in seven different combinations.

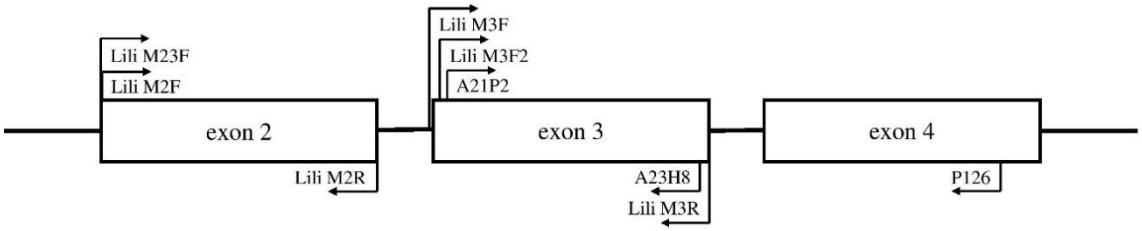



**Fig. 1** – Schematic representation of MHC-I (exon 2 intron 2, exon 3, intron 3, exon 4 and intron 4) where arrows indicates the position of the primers used in the present study. Forward primers are represented above each exon (Lili M23F, Lili M2F, Lili M3F, Lili M3F2 and A21P2), while the reverse primers are showed below (Lili M2R, A23H8, Lili M3R and P126). Primers A21P2 and P126 were originated from Strandh et al. (2011), while primer combo LiliM3F and LiliM3R was used for MiSeq Illumina sequencing.

*MiSeq Illumina sequencing on MHC-I exon 3*

The MHC-I exon 3 primers Lili M3F (5´-TCGYGTTCCAGGGGCTCACA–3´) and Lili M3R (5´-GGCYGTGCTGGAGAGGAAA–3´) were designed for godwits used for MiSeq Illumina MHC-I genotyping. The primer pair satisfactorily amplified a 247 bp long fragment in all 84 godwit individuals. Each 25 µl amplicon PCR reaction contained 12.5 µl 2X Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) and 0.5 µM of each primer and 25 ng of gDNA template. The two step PCR profile was set to 25 cycles 98°C (10 s) and 72°C (15 s), ending with 72°C for 10 minutes. PCR products were checked on a 2% agarose gel and then cleaned with Agencourt AMPure XP-PCR Purification kit (Beckman Coulter, Indianapolis, USA) following manufacturer´s instructions. PCR clean-up was done by adding 20 µl AMPure XP beads to each reaction, washing with 75% ethanol and adding 43 µl of $ddH_2O$ for elution.

To allow recovery of individual amplicons after demultiplexing a unique combination of forward and reverse Illumina index was added to each sample by using Nextera XT v2 Index Kit (Illumina Inc., San Diego, CA, USA). A 25 µl PCR reactions volume were prepared

containing 12.5 µl 2X Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 3 µl of each index primers and 2 µl of cleaned PCR amplicon product. The PCR profile was set to eight cycles at 98°C (10 s), 55°C (30 s) and 72°C (15 s), ending with 72°C for 5 minutes and PCR products were checked on a 2% agarose gel and cleaned with Agencourt AMPure XP-PCR Purification kit (Beckman Coulter, Indianapolis, USA). Cleaning was done as mentioned above, except for the addition of 23 µl AMPure XP beads to each sample and 38 µl of $ddH_2O$ for elution. Cleaned PCR products were checked on a 2% agarose gel and concentration was measured with Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific/Invitrogen, Waltham, USA) modified for a 96 well plate. Each plate was equimolarly pooled and quantified with Qubit (Thermo Fisher Scientific, Waltham, USA) and run on Bioanalyzer DNA 2100 chip for quality and size validation. All pools were equimolarly pooled together to a final 4 nM library and sent for 300 bp paired-end Illumina MiSeq sequencing (Illumina Inc., San Diego, CA, USA) at the DNA sequencing facility Department of Biology, Faculty of Science, Lund University.

*Filtering MiSeq Illumina data*

For bioinformatics post-processing of HTS data we used Amplicon Sequencing Analysis Tools (AmpliSAT) (web server http://evobiolab.biol.amu.edu.pl/amplisat/; Sebastian et al. 2016) for demultiplexing, clustering and filtering. Clustering and filtering parameters for removing artefacts from our dataset was done in four steps having in mind key assumptions based upon previously described methods (Galan et al. 2010; Lighten et al. 2014; Stutz and Bolnick 2014). Step 1) in order to make sure that each amplicon had a reliable read depth for allele characterization a linear plot of amplicon read depths was produced. This allowed us to detect and remove poor quality amplicons (*i.e.* with low read depth) that could introduce bias into the analysis. The minimum required read depth was set to 4000 reads per amplicon. Step 2) in order to reassign reads from artefacts to the parental sequences they arose from we used the clustering function in Amplisas (Sebastian et al. 2015). Artefact sequences were merged with the dominant sequences when they varied by 1-2 bp and had ≤ 25% of read depth compared to the dominant sequences. Sequences that varied by 1-2 bp from the dominant sequences but with higher read depth than 25% were classified as

"subdominants" and formed a new cluster. Step 3) for the establishment of a suitable per amplicon frequency, since there is no information regarding the number of MHC-I loci for godwits, the threshold was determined by the best match between technical replicates (n=6 samples) in a similar fashion to Karlsson and Westerdahl (2013) and O´Connor et al. (2016). Best matches were obtained with a per amplicon frequency of 3.4% and any sequences occurring below this value were considered artefacts and removed. Finally, during step 4) only sequences that differed 3bp from the expected length (247 bp) were kept. For more details, see online resource Material and Methods.

## Data analysis

*Validation and identification of putative functional alleles*

MHC-I alleles from cloning were considered verified when found in two independent PCR reactions and/or when sequences matched those found by MiSeq Illumina for the same individual. All MHC-I alleles from MiSeq Illumina that remained in the data set after the filtering were considered verified. All verified alleles from cloning and Illumina sequencing were blasted in GenBank to confirm that they were MHC-I alleles and to check if they had been described previously in any other avian species. Unique alleles were uploaded to the GenBank database and given species-specific names, following the Klein et al. (1990) nomenclature for naming MHC alleles (*Mhc*Lili-UA*xx). After manual alignment of alleles in BioEdit 7.2.5 Sequence Alignment Editor the sequences were translated to amino acid sequences, all in open reading frame.

*Analysis of allelic polymorphism, recombination and positive selection in godwits*

Analyses on polymorphism of MHC exon 3 sequences from godwits were performed in MEGA 7.0.14 (Kumar et al. 2016). These analyses included estimation of the number of polymorphic amino acid sites ($S_{aa}$), average nucleotide diversity ($\pi$), evolutionary divergence for nucleotide sequences ($d_{nt}$) and amino acid sequences ($d_{aa}$), the two were estimated using the Kimura 2-parameter (K2P) model (Kimura 1980) and p-distance model,

respectively. The models were run with a gamma distribution (α=1) and with uniform rates, respectively, and with 1000 bootstrap repeats. All analyses were carried out either for all alleles simultaneously (*i.e.* nucleotide alleles with and without a 3bp deletion; n=47), for full length alleles only (nucleotide alleles, n=38) or for alleles with a 3bp deletion alleles only (n=9) (corresponding n-values for amino acid sequences were 40, 31 and 9). To allow an overview of allele frequency on the godwit population, histograms of mean allele frequencies were created with SigmaPlot (Systat Software, San Jose, CA) (online resource Fig. S1).

Average rates of synonymous ($d_S$) and nonsynonymous ($d_N$) substitutions in the PBR and non-PBR regions were also estimated in MEGA 7.0.14 (Kumar et al. 2016). To determine evidence of selection in the PBR and non-PBR rates of $d_N/d_S$ and codon-based Z test were calculated according to the Nei-Gojobori method with a Jukes-Cantor correction and 1000 bootstrap replicates for variance estimation. These analyses were carried out either for all alleles simultaneously (with and without a 3bp deletion; n=44, the n-value dropped from 47 to 44 since 2 bp in the beginning of the sequence and 2 bp in the end of the sequence were encoding incomplete codons) or for full length alleles only (n=35) or for alleles with a 3bp deletion alleles only (n=9). The full PBR region was inferred in accordance with the previously documented PBR sites from human and chicken MHC-I (Wallny et al. 2006) which overlap with the described patterns of positive selection in other bird species (*e.g.* Alcaide et al. 2009; Cloutier et al. 2011; Buehler et al. 2013). The α1 (exon 2) PBR codons were the following 5, 7, 9, 24, 25, 34, 43, 58, 62, 65, 66, 68, 69, 72, 73, 75, 76, 79, 80, 83, while in α2 (exon 3) they were 96, 98, 112, 114, 121, 141, 144, 145, 149, 151, 154, 155, 158, 159, 162, 166 and 170 (Fig. 2). Inference of positive selection analysis with fixed-site models were run only on exon 3 alleles using 12 PBR positions (Fig. 3) excluding main chain-binding sites (codons: 141, 144, 145, 158 and 170) which are highly conserved and responsible for anchoring the N- (also called A pocket) and C- (F pocket) termini of the peptides (Bjorkman et al. 1987; Saper et al. 1991; Wallny et al. 2006).

Before testing for evidence of positive selection with Maximum Likelihood (ML) methods we explored the presence of recombination breakpoints using the genetic algorithm for recombination detection (GARD), available through the Datamonkey webserver (www.datamonkey.org; Pond et al. 2006; Delport et al. 2010). These analyses

were done for all alleles, with and without a 3bp deletion, (n=40 randomly selected nucleotide sequences out of 44; 40 is the maximum allowed number of sequences for REL analysis), for full length alleles (n=35) or for alleles with a 3bp deletion (n=9). Inferred GARD trees were then used to perform analysis of positive selection with ML methods. It is not always that a priori fixed-site model describe best positively selected sites since selection (positive *vs.* negative) might act outside the inferred PBR, particularly so in non-model species (Yang and Swanson 2002). Random-sites models, like ML, can thus be better at identifying positively selected sites once it describe the overall variation among sites (Furlong and Yang 2008). Evidence for positive selection was therefore done by combining the results of the following ML methods: single-likelihood ancestor counting (SLAC) at P=0.1, fixed effects likelihood (FEL) at P=0.1 and random effects likelihood (REL) with BF > 50 (online resource Table S2), all implemented on the Datamonkey webserver (Pond et al. 2006; Delport et al. 2010).

*Neighbour-network and phylogenetic analysis on exon 3 alleles from three Charadriiformes species*

MHC-I exon 3 sequences from putatively classical alleles were compared between godwits, knots and gulls. We were able to retrieve 21 knot sequences (Caca-UA*01-02; Caca-UA*04; Caca-UA*06-09; Caca-UA*11-15; Caca-UA*17-25; Buehler et al. 2013) and 21 gull sequences (Lasc-UAA*01; Lasc-UAA*03-16; Lasc-UBA*02-06; Lasc-UBA*08; Cloutier et al. 2011) from GenBank, while for godwits we took 21 random MHC sequences. Analysis of allelic polymorphism for the three species was calculated as described above.

Phylogenetic networks allows the representation of alternative phylogenetic histories and besides the assumption of mutation and speciation events also takes into account gene loss, duplication and recombination events (Bryant and Moulton 2004) processes which are known to affect the MHC gene evolution (Nei et al. 1997; Hess and Edwards 2002; Spurgin et al. 2011). Neighbour-net phylogenetic network on exon 3 alleles of Charadriiformes members were built using SplitsTree v.4.14.4 (Huson and Bryant 2006) based on the substitution model KP2. Best-fit nucleotide substitution model was determined

with JModel test 2.1.10 (Posada 2008). Thirty-two exon 3 MHC-I alleles representing locus-specific clades from knots (Caca-UA*01-02; Caca-UA*04; Caca-UA*06-09; Caca-UA*11-15; Caca-UA*17-25; Caca-UA*26-36; Buehler et al. 2013) and gulls (Lasc-UAA*01; Lasc-UAA*03-16; Lasc-UBA*02-06; Lasc-UBA*08; Lasc-UCA*01-04; Lasc-UDA*01-04; Lasc-UDA*06; 08 and 09; Cloutier et al. 2011) were downloaded from GenBank and used for building species specific neighbour-net phylogenetic networks. The godwit network was built using full length exon 3 sequences (276 bp; Lili-UA*01-04) and partial exon 3 sequences (240 and 243 bp; Lili-UA*05-47). For godwits the model was run with a transition/transversion (R) rate of 1.9421, probability of invariable sites of 0.4470 (p-inv) and a gamma distribution shape parameter (α) of 0.4250. For knots, the model was run with R rate of 1.3753, p-inv of 0.3220 and an α of 0.5000, while for gulls we used a R rate of 1.4271, p-inv of 0.5500 and an α of 0.5750. In all networks 1000 bootstrap repeats were used.

The phylogenetic relationships of MHC-I alleles within Charadriiformes (godwits, knots and gulls) was studied using ML trees in MEGA 7.0.14 (Kumar et al. 2016). Selection of the best substitution models was performed by BIC and AICs in MEGA and phylogenetic analyses were done simultaneously for exon 2-3 and separately for exon 4, using a chicken MHC-I sequence as outgroup. Nucleotide based trees were built using T92 models (Tamura1992), while amino acid trees were inferred by JTT models (Jones et al. 1992), both using 1000 bootstrap replication.

# Results

*Black-tailed godwit MHC-I, the α1, α2 and α3 domains*

Sanger sequencing using nine different primers on both gDNA and cDNA from a single godwit individual resulted in 100 confirmed MHC-I sequences of different lengths (174–741 bp; online resource Table S3). The long transcripts (736-738 bp) allowed verification of four distinct MHC-I alleles: Lili-UA*01, Lili-UA*02, Lili-UA*03 and Lili-UA*04 (GenBank access numbers: KY351552- KY351555). These transcripts cover the major part of exon 2-4; partial α1 (82 out of 88 amino acids), entire α2 (92 amino acids) and partial α3 (72 out of 91 amino

acids). The godwit MHC-I amino acid sequences could easily be aligned to MHC sequences from other birds (Fig. 2). Lili-UA*01 and Lili-UA*02 have a 3bp deletion at nucleotide positions 415-417 in the α2 domain (amino acid position 147 in the alignment) while Lili-UA*03 and Lili-UA*04 are full length. Classical class I amino acid sequences have particular highly conserved sites such as the peptide main-chain domain and in the godwit MHC-I transcripts there is no deviation from the consensus "YRTKWYY" sequence (in non-mammalian vertebrates) except for Lili-UA*03 that has a non-conservative substitution of tyrosine (Y) to histidine (H) at position 170 (Betts and Russell 2003). Inter and intra domains are also highly conserved across vertebrates, *e.g.* the cysteine (C) residues responsible for the disulphide bridge formation (Grossberger and Parham 1992). In the godwit transcripts 12 out of the 18 inter and intra domains were covered and out of these 11 residues remained unaltered, while one position (position 10) was highly variable (Fig. 2). The CD8 binding region (positions 218-227 and 244-255 in the alignment) is known to be highly variable between species but conserved within species (Salter et al. 1989; Kaufman et al. 1994) and godwits showed no deviation to this rule with only allele Lili-UA*03 exhibiting a conservative substitution of a lysine (K) instead of asparagine (N) (Betts and Russell 2003). Gene expression was estimated in this single godwit individual and five alleles (Lili-UA*01-05) out of seven in total (Lili-UA*01-07) were transcribed (found in RNA).

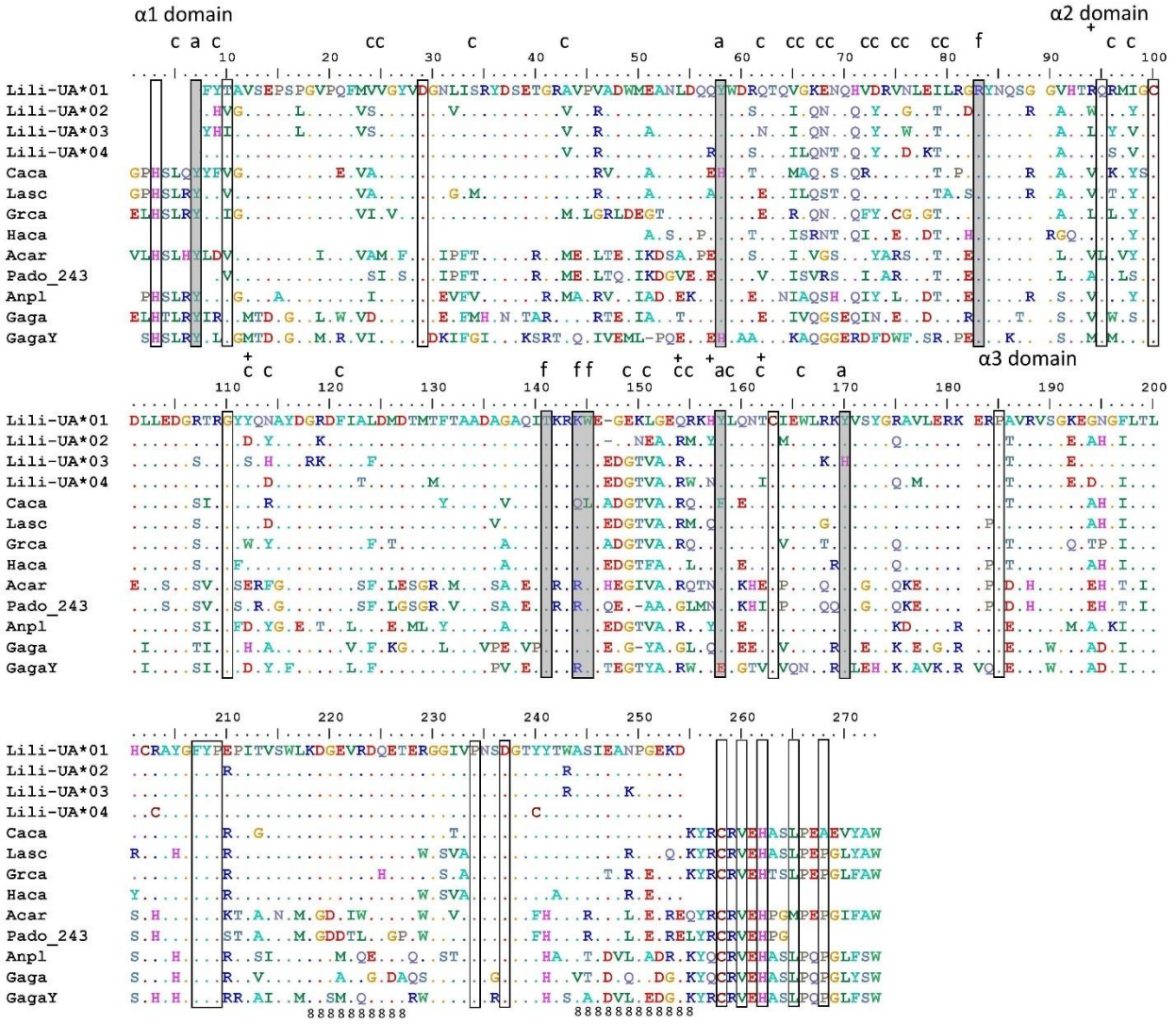



**Fig. 2 –** Amino acid alignment of Icelandic black-tailed godwits (*Limosa limosa islandica;* Lili) MHC-I alleles covering the α1, α2 and α3 regions (the first residue of the alignment is the first residue in α1). Identity to Lili-UA*01 is indicated by dots, a blank space determines the end of an exon and dashes indicate true gaps. Transparent-boxes indicate intra and inter domain contacts, antigen-peptide main-chain binding residues are indicated by light-grey boxes, "c" represents non-main chain peptide contacts, "a" and "f" indicates the A and F pocket respectively (anchoring sites of the two ends of the peptide), (Wallny et al. 2006) and "8" indicates the CD8 binding sites (Bjorkman et al. 1987; Saper et al. 1991). NCBI sequence sources are: Caca (red knot, *Calidris canutus*, KC205116.1), Lasc (red-billed gull, *Larus scopulinus*, HM025963.1), Grca (florida sandhill crane, *Grus canadensis pratensis*, AF033106.1), Haca (blue petrel, *Halobaena caerulea*, JF276884.1), Acar (great reed warbler, *Acrocephalus arundinaceus*, AJ005503.1), Pado_243 (house sparrow, *Passer domesticus* with 3bp deletion, not submitted), Anpl (mallard duck, *Anas platyrhynchos*, GU245878.1), Gaga (chicken, *Gallus*

*gallus domesticus* BF2, HQ141386.1), GagaY (chicken, *Gallus gallus domesticus* YF6, XM_003643736.2).

*Exon 3 sequences (α2 domain) from MiSeq Illumina*

The average read depth per amplicon after filtering the MiSeq data was 11,322 ± 335 ($\bar{x}$ ± SE). The sequences from 90 amplicons (n=84 individuals; n=6 replicates) were either 240 or 243 bp and genotyping repeatability across replicates was 100%. The exon 3 sequences covered 80 out of the 91 amino acids in the α2 domain. Forty-seven MHC-I alleles were verified (KY351556-KY351598) and nine of these had a 3bp deletion at amino acid position 147 (hereafter 3bp deletion alleles). In the individual screened with both Sanger sequencing and MiSeq Illumina sequencing, six out of seven alleles were found with both techniques (Lili-UA*02 to UA*07) but the seventh allele (Lili-UA*01) was not found using MiSeq. The missing allele Lili-UA*01 was found in very low read depth in the MiSeq amplicon data and was therefore deleted during the filtering process.

The 47 MHC-I alleles from Illumina MiSeq translated into 40 unique amino acid sequences and more than half (10 out of 18) of the segregating sites were found within the PBR (Fig. 3). Functional characteristics of the inter- and intra-domains and main-chain binding sites were highly conserved across all alleles, with the exception of tyrosine (Y) at position 170 that was replaced by histidine (H) in four alleles (UA*03, UA*08, UA*09 and UA*14) and by phenylalanine (F) in six alleles (UA*23-24, UA*36, UA*39 and UA*44-45), a pattern seen also in the long transcripts (Fig. 2). Motifs that were unique for the 3bp deletion alleles and full length alleles are *e.g*. “GENE” (at position 148-151) and “EDGTVA” (positions 147-152), respectively (Fig. 3).

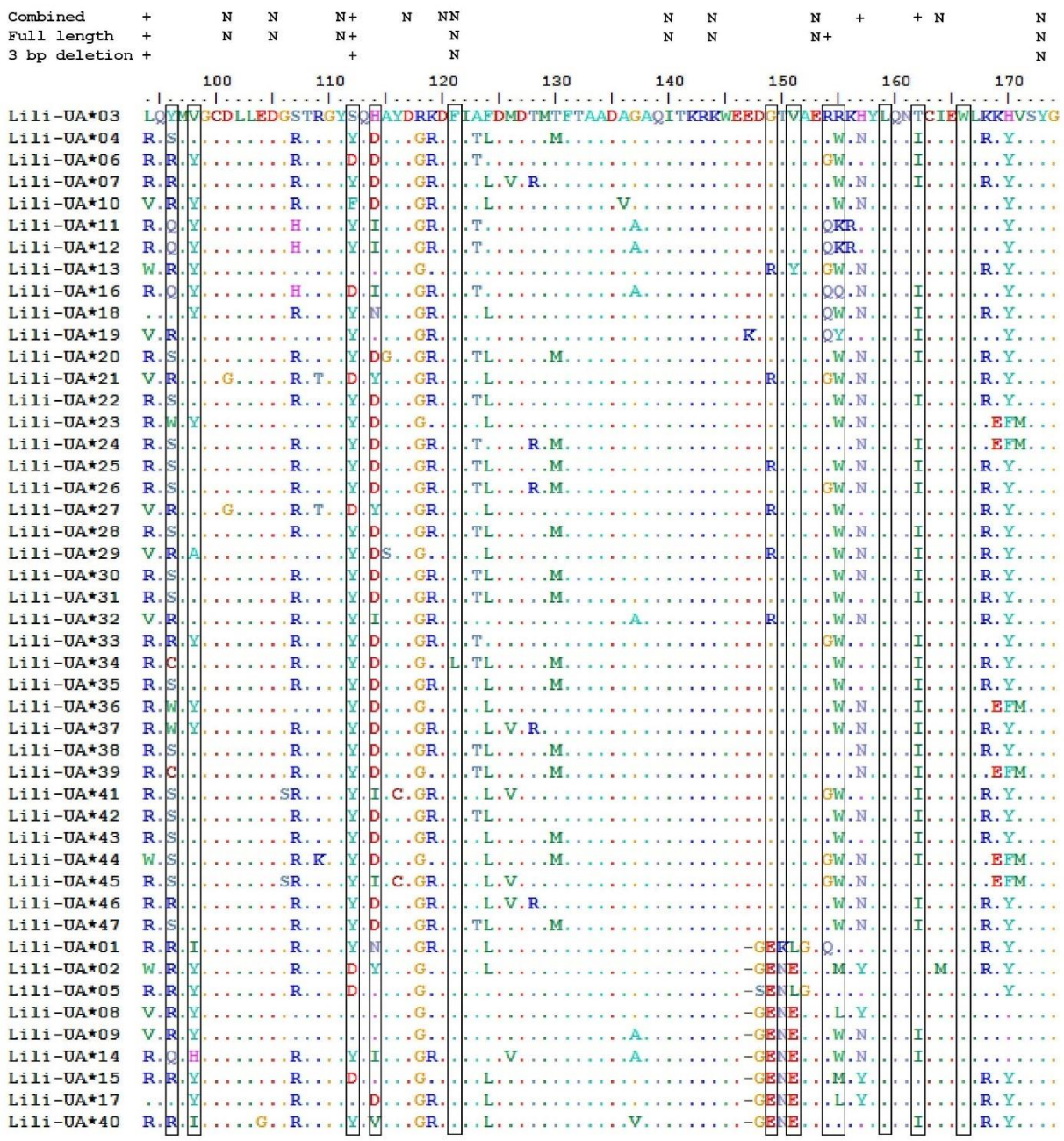



**Fig. 3 –** Alignment of Icelandic black-tailed godwit MHC-I alleles covering 240-243 bp of the α2 region (exon 3) sequenced with MiSeq Illumina. Identity to Lili-UA*03 is indicated by dots and dashes indicate gaps. Alleles Lili-UA*01, 02, 05, 08, 09, 14, 15, 17 and 40 have a 3bp deletion at position 147. Boxes indicate PBR sites inferred from Wallny et al. (2006) excluding main-chain binding sites. Residues under positive selection (+), negative selection (N) were calculated using SLAC, FEL and REL methods (www.datamonkey.org; Pond et al. 2006; Delport et al. 2010).

*Exon 3 allelic diversity and frequency*

There were two to seven alleles in total per individual ($\bar{x}$ ±SD: 4.92 ±1.09) and assuming heterozygosity godwits have between one and four MHC-I loci. Among the 84 genotyped individuals all individuals had full length alleles (1-6 per individual; $\bar{x}$ ±SD: 3.74 ±1.11), 18 individuals had only full length alleles (2-6 per individual; $\bar{x}$ ±SD: 4.39 ±0.92) and 66 individuals had at least one 3bp deletion allele (1-3 per individual; $\bar{x}$ ±SD: 1.50 ±0.64). Regarding allele frequency distribution, full length alleles seem to range from common to rare while 3bp deletion alleles are overall more common and have a more even distribution in the population dataset (online resource Fig. S1).

*Polymorphism, inference of positive selection and recombination in exon 3 alleles*

There were no striking differences in polymorphism between full length and 3bp deletion alleles, though 3bp deletion alleles have slightly higher nucleotide diversity ($\pi$) and divergence (nucleotide and amino acid distances (Table 1). Fixed-site model analysis showed no significant support for positive selection acting on the PBR of exon 3, though the dN/dS ratio was twice as high in the PBR relative to the non-PBR (Table 1). The absence of positive selection in the PBR of the godwit MHC alleles is due to high rates of synonymous substitutions (dS), particularly so for the 3bp deletion alleles. Selection estimation by ML methods on the combined alleles (with and without 3bp deletion) identified four sites subject to positive selection in exon 3 (positions 94, 112, 157 and 162 in Fig. 3) and two of these fall outside the PBR inferred by Wallny et al. (2006). When this analysis was run on 35 full length alleles only, it identified three positively selected sites (positions 94, 112 and 154), two positions overlap with the combined analysis, but position 157 and 162 was lost and position 154 in the PBR added. When running the ML analysis on nine 3bp deletion alleles two already identified positively selected sites were found, one inside and one outside PBR (position 94 and 112).

Recombination was investigated separately for the full length and 3bp deletion alleles. There were no recombination points in the full length alleles but GARD predicted one recombination point at amino acid position 121 in the nine 3bp deletion alleles.

Recombination events were also detected in the 3bp deletion alleles using SplitsTree Phi test (data not shown).

**Table 1 –** Measures of polymorphism (polymorphic amino acid sites ($S_{aa}$) and average nucleotide diversity ($\pi$)) and evolutionary divergence (for nucleotide sequences ($d_{nt}$) and amino acid sequences ($d_{aa}$)) of MHC class I exon 3 alleles in the Icelandic black-tailed godwit (*Limosa limosa islandica*). These analyses were carried out either for all alleles simultaneously (*i.e.* nucleotide alleles with and without a 3bp deletion; n=47), for full length alleles only (nucleotide alleles, n=38) and for alleles with a 3bp deletion alleles only (n=9) (corresponding n-values for amino acid sequences were 40, 31 and 9). Estimates of non-synonymous ($d_N$), and synonymous ($d_S$) substitution rates, plus the $d_N/d_S$ ratio, was calculated separately for the PBR and non-PBR regions (the PBR was defined as 12 amino acids, see Fig. 3), for all alleles simultaneously (n=44), for full length alleles only (n=35) and for alleles with a 3bp deletion alleles only (n=9).

| | dN±SE | dS±SE | dN/dS | Saa | π | dnt±SE | daa±SE |
|---|---|---|---|---|---|---|---|
| **All** | | | | | | | |
| **PBR** | 0.322±0.104 | 0.310±0.160 | 1.039 | 10 | 0.255 | 0.560±0.285 | 0.422±0.075 |
| **Non-PBR** | 0.046±0.010 | 0.082±0.025 | 0.561 | 27 | 0.041 | 0.044±0.016 | 0.103±0.020 |
| **Full length** | | | | | | | |
| **PBR** | 0.239±0.086 | 0.209±0.148 | 1.144 | 10 | 0.220 | 0.438±0.245 | 0.335±0.067 |
| **Non-PBR** | 0.040±0.009 | 0.059±0.022 | 0.678 | 23 | 0.039 | 0.043±0.016 | 0.091±0.019 |
| **3bp deletion** | | | | | | | |
| **PBR** | 0.302±0.107 | 0.394±0.179 | 0.766 | 8 | 0.375 | 0.505±0.310 | 0.375±0.093 |
| **Non-PBR** | 0.041±0.012 | 0.078±0.027 | 0.526 | 14 | 0.089 | 0.041±0.019 | 0.089±0.023 |

*Polymorphism and phylogenetic allelic relationships among three Charadriiformes species*

When comparing the godwit exon 3 polymorphism with that of two other Charadriiformes species, the knot and the gull, the godwit polymorphism measures mostly fall in between the two other species (Table 2). However, the signal of positive selection in the PBR was less pronounced for godwits than for either gulls or knots. The godwit MHC-I organization was more similar to the knots than to the gulls, both knots and godwits for example have putatively classical MHC-I alleles with and without a 3bp deletion. Interestingly, both knot

and gulls have reported non-classical genes though we did not find any evidence of non-classical genes in the godwit (Table 3).

**Table 2 –** Measures of polymorphism (polymorphic nucleotide ($S_{nt}$) and amino acid sites ($S_{aa}$)) and average nucleotide diversity ($\pi$) of MHC class I exon 3 alleles in Icelandic black-tailed godwits (*Limosa limosa islandica*), red-billed gulls (*Larus scopulinus*) and red knots (*Calidris canutus*). All analyses were carried out on 21 putatively classical alleles per species. Estimates of non-synonymous ($d_N$) and synonymous ($d_S$) substitution rates, plus the $d_N/d_S$ ratio, was calculated separately for the PBR and non-PBR regions (the PBR was defined as 12 amino acids, see Fig. 3).

| α2 | **dN±SE** | **dS±SE** | **$d_N/d_S$** | **$S_{nt}$** | **$S_{aa}$** | **π** |
|---|---|---|---|---|---|---|
| **Black-tailed godwit** | | | | | | |
| PBR | 0.340±0.113 | 0.344±0.172 | 0.988 | 8 | 9 | 0.268 |
| Non-PBR | 0.047±0.011 | 0.093±0.030 | 0.505 | 10 | 22 | 0.042 |
| **Red-billed gull** | | | | | | |
| PBR | 0.236±0.108 | 0.179±0.118 | 1.318 | 5 | 7 | 0.191 |
| Non-PBR | 0.037±0.010 | 0.057±0.020 | 0.649 | 7 | 17 | 0.028 |
| **Red knot** | | | | | | |
| PBR | 0.428±0.170 | 0.271±0.132 | 1.579 | 7 | 7 | 0.320 |
| Non-PBR | 0.072±0.013 | 0.145±0.032 | 0.497 | 19 | 35 | 0.055 |

**Table 3 –** Number of putatively classical and non-classical MHC-I alleles in three species within the order Charadriiformes. Range of loci and allele numbers (given in brackets) identified per individual, within a sample of 84 Icelandic black-tailed godwits (*Limosa limosa islandica*) and 8 red knots (*Calidris canutus*). For the red-billed gull (*Larus scopulinus*), individual information regarding number of alleles was not available and instead information refers to the total number of loci identified across a sample of 470 individuals.

| **Species** | **Classical alleles** | | | | **Non-classical alleles** |
|---|---|---|---|---|---|
| | *Total* | *3bp del* | *Full length* | *Minor* | |
| **Black-tailed godwit**[a] | 1-4 (2-7) | 0-2 (0-3) | 1-3 (1-6) | 0 | 0 |
| **Red knot**[b] | 2-4 (3-8) | 0-2 (0-3) | 1-3 (2-5) | 0 | 1-2 (2-4)[c] |
| **Red-billed gull**[d] | 2 | 0 | 1 | 1 | 2 |

[a]This study
[b]Buehler et al. (2013)
[c]One of the genes could possibly be a pseudogene
[d]Cloutier et al. (2011)

We compared phylogenetic neighbour network built on MHC-I exon 3 alleles between the three Charadriiformes species. In the godwit network, the MHC-I alleles formed a single undifferentiated cluster, though the 3bp deletion alleles clustered together (bootstrap=61.3) separate from full length alleles (Fig. 4a). Unlike the godwit, the networks of knot and gull MHC-I alleles displayed high bootstrap support for some clusters, particularly so for putatively non-classical alleles, but also for a few clusters of putatively classical alleles that can be found at the core of both networks (Fig. 4b, 4c). One similarity between the godwit and knot networks is that the 3bp deletion alleles clustered separately with rather high bootstrap support, 61.3 and 87.5 for godwits and knots, respectively.

Phylogenetic reconstructions of amino acid sequences based on exons 2-3 and 4, respectively, resulted as expected in trees with different features and bootstrap support. The phylogenetic reconstruction of exons 2 and 3, the regions that hold the PBR, had low bootstrap support in the deeper nodes and significant support was only found in a few terminal nodes. This tree placed one non-classical gull MHC-I allele among the godwit alleles and another non-classical gull MHC-I allele among non-classical knot alleles (Fig. 5a,

nucleotide tree in online resource Fig. S2a). Interestingly, the two 3bp deletion alleles in godwits were found distant in the tree and not clustered as in the network analysis. A gene tree that perfectly matched the species tree appeared when the phylogenetic reconstruction was based on exon 4 amino acid sequences from godwits, gulls and knots. Exon 4 encodes a structural part for the MHC molecule and the bootstrap support was considerably better in this tree (Fig. 5b; nucleotide tree in online resource Fig. S2b).

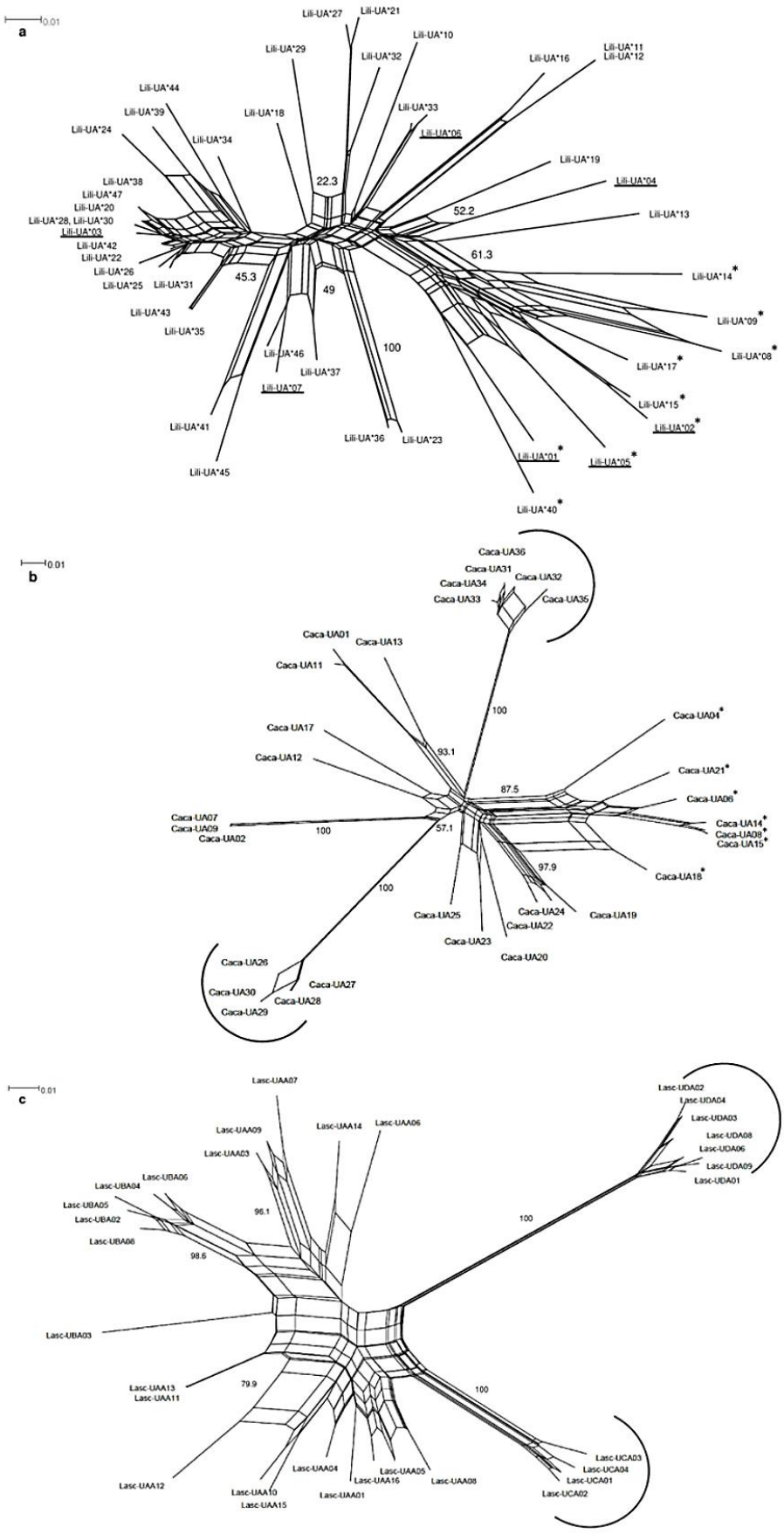

a
0.01
Lili-UA*27
Lili-UA*21
Lili-UA*10
Lili-UA*29
Lili-UA*32
Lili-UA*16
Lili-UA*11
Lili-UA*12
Lili-UA*44
Lili-UA*39
Lili-UA*18
Lili-UA*33
Lili-UA*24
Lili-UA*34
Lili-UA*06
Lili-UA*19
Lili-UA*38
Lili-UA*04
Lili-UA*47
Lili-UA*20
Lili-UA*28, Lili-UA*30
Lili-UA*03
Lili-UA*42
Lili-UA*22
Lili-UA*26
Lili-UA*25
Lili-UA*31
Lili-UA*43
Lili-UA*35
Lili-UA*46
Lili-UA*37
Lili-UA*07
Lili-UA*41
Lili-UA*45
Lili-UA*36
Lili-UA*23
Lili-UA*01*
Lili-UA*05*
Lili-UA*40*
Lili-UA*13
Lili-UA*14*
Lili-UA*09*
Lili-UA*08*
Lili-UA*17*
Lili-UA*15*
Lili-UA*02*
22.3
52.2
61.3
45.3
49
100
b
0.01
Caca-UA36
Caca-UA31
Caca-UA34
Caca-UA32
Caca-UA33
Caca-UA35
Caca-UA01
Caca-UA13
Caca-UA11
Caca-UA17
Caca-UA12
Caca-UA04*
Caca-UA21*
Caca-UA06*
Caca-UA07
Caca-UA09
Caca-UA02
Caca-UA14*
Caca-UA08*
Caca-UA15*
Caca-UA18*
Caca-UA25
Caca-UA24
Caca-UA19
Caca-UA22
Caca-UA23
Caca-UA20
Caca-UA26
Caca-UA27
Caca-UA30
Caca-UA28
Caca-UA29
100
93.1
87.5
57.1
97.9
c
0.01
Lasc-UAA07
Lasc-UAA09
Lasc-UAA14
Lasc-UAA06
Lasc-UAA03
Lasc-UDA02
Lasc-UDA04
Lasc-UDA03
Lasc-UDA08
Lasc-UDA06
Lasc-UDA09
Lasc-UDA01
Lasc-UDA05
Lasc-UBA04
Lasc-UBA05
Lasc-UBA02
Lasc-UBA08
Lasc-UBA03
Lasc-UAA13
Lasc-UAA11
Lasc-UAA12
Lasc-UAA10
Lasc-UAA15
Lasc-UAA04
Lasc-UAA05
Lasc-UAA16
Lasc-UAA08
Lasc-UAA01
Lasc-UCA03
Lasc-UCA04
Lasc-UCA01
Lasc-UCA02
96.1
98.6
79.9
100

**Fig. 4 -** Neighbour-net phylogenetic network of MHC-I exon 3 alleles from: (a) Icelandic black-tailed godwits (*Limosa limosa islandica*), (b) Red knot (*Calidris canutus*) and (c) Red-billed gull (*Larus scopulinus*). The 47 Icelandic black-tailed godwits alleles represented are either full (276 bp) and partial (240 bp) lengths. Alleles found with both Sanger and Illumina screening of a single individual are underlined. 32 Red knot as well as 32 Red-billed gull nucleotide alleles were downloaded from Genbank. Alleles with a 3bp deletion are indicated by a star "*", brackets indicate clades with alleles that have putatively non-classical or pseudogene function (for more details see Buehler et al. 2013; Cloutier et al. 2011). Bootstrap values after 1000 repeats for main splits are represented.

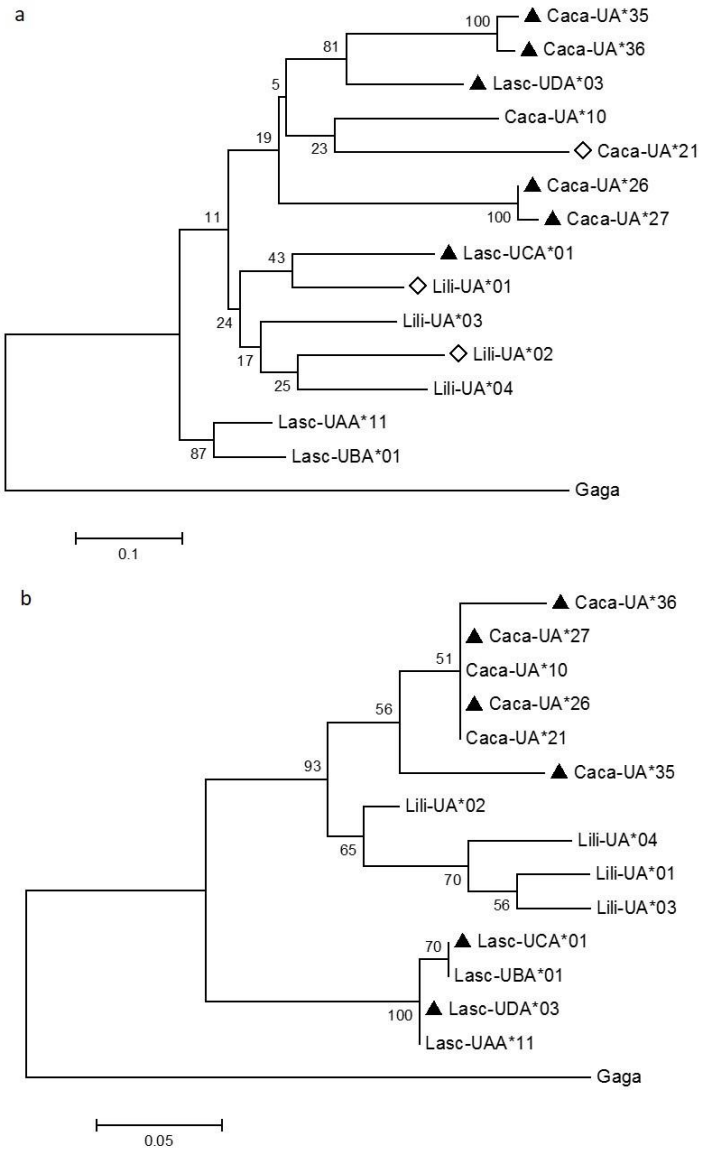



**Fig. 5 –** Amino acid phylogenetic reconstruction of MHC-I from three Charadriiformes species; the Icelandic black-tailed godwit (Lili, *Limosa limosa islandica*), the red knot (Caca, *Calidris canutus*) and red-billed gull (Lasc, *Larus scopulinus*) using Maximum Likelihood with domestic chicken (Gaga, *Gallus gallus domesticus*) as an outgroup. Trees were done separately for (a) exon 2 and 3 holding the PBR, and (b) for exon 4 encoding the structural part of MHC. Putatively classical alleles in the three species are: Lasc-UAA*11 (HM008713.1), Lasc-UBA*01 (HM008714.1), Caca-UA*10 (KC205115.1), Caca-UA*21 (KC205126.1) and Lili-UA*01 to Lili-UA*04. The remaining alleles are non-classical (marked with a black triangle): Lasc-UCA*01 (HM008715.1), Lasc-UDA*03 (HM008716.1), Caca-UA*26 (KC205131.1), Caca-UA*27 (KC205132.1), Caca-UA*35 (KC205140.1) and Caca-UA*36 (KC205141.1). On exon 3 Lili-UA*01, Lili-UA*02 and Caca-UA*21 have a 3bp deletion (marked with

a white diamond), while Caca-UA*26 and Caca-UA*27 have a 12bp insertion. Numbers on branches indicates bootstrap values after 1000 repeats.

# Discussion

In this study MHC-I was partly characterized in a long-distance migrant wader, the Icelandic black-tailed godwit. We isolated four long transcripts from a single individual, covering the major part of the extra-cellular MHC-I protein. MHC-I exon 3 was genotyped successfully in 84 individuals using MiSeq Illumina and in total we could verify 47 different alleles, *i.e*. exon 3 sequences. Godwits have between one and seven putatively classical MHC-I alleles in open reading frame per individual and our preliminary data suggests that at least five alleles are expressed. We did not detect any putatively non-classical genes as have previously been reported in two other Charadriiformes species, the knot and the gull.

The godwit MHC-I transcripts (733-736 bp) showed typical characteristics of functional antigen-presenting genes *e.g.* highly conserved peptide main-chain domain, intra and inter domain and CD8 binding sites (Grossberger and Parham 1992; Halenius et al. 2015). The only exception was the godwit allele Lili-UA*03 where tyrosine (Y) had been replaced by histidine (H) at position 170, a peptide anchoring site. This substitution is likely to have an impact on the MHC protein since the amino acids tyrosine and histidine have very different chemical properties, tyrosine is aromatic and histidine is basic (Betts and Russell 2003). Nevertheless, the same substitution (Y to H) in the same peptide anchoring position has been found in classical MHC genes in humans, mice and knots and seems to not affect the function though most likely the binding properties of the MHC protein (Shum et al. 1999; Buehler et al. 2013). When examining this position in 40 exon 3 alleles from MiSeq Illumina we found four more alleles where tyrosine (Y) was substituted by histidine (H) and also six alleles where tyrosine (Y) was substituted with phenylalanine (F). The substitution to phenylalanine is conservative since an aromatic amino acid is replaced by another aromatic amino acid (Betts and Russell 2003). A tyrosine replacement by phenylalanine, in the same peptide anchoring position, has also been observed in other birds, mallards and knots, but was then associated with putatively non-classical alleles in both these species (Moon et al.

2005; Buehler et al. 2013). We could not find any characteristics suggesting the occurrence of non-classical MHC-I alleles in godwits.

The class I alleles in godwits are of two different lengths, one codon is absent in exon 3 in some alleles. These 3bp deletion alleles were detected with both Sanger sequencing and MiSeq Illumina and they tended to have a more even distribution in the godwit population than full length alleles. Most individuals (79%) had both full-length and 3bp deletion alleles. Alleles with 3bp deletions are frequently reported among birds of the order Passeriformes (Alcaide et al. 2013; O'Connor et al. 2016). Within the order Charadriiformes a 3bp deletion, possibly at the same position as in godwits, was reported in classical MHC-I genes in the phylogenetically close knot but not in the more distant gull (Cloutier et al. 2011; Buehler et al. 2013). The 3bp deletion alleles have some unique motifs that are not found in the full length alleles, *e.g.* "GENE" at position 148-152, likewise the full length alleles have unique motifs that are not found in the 3bp deletion alleles, *e.g.* "EDGTVA" at position 147-152. Interestingly, these two motifs are also found in the 3bp deletion alleles and full length alleles of knots (Buehler et al. 2013). Since both types of alleles are found in godwits and knots but not in gulls it could indicate that full length and 3bp deletion alleles were present in their *Scolopacidae* common ancestor. However, neither the phylogenetic reconstruction on exons 2 and 3 nor a network analyses based on exon 3 sequences from godwits and knots (data not shown) support that the 3bp deletion alleles in godwits and knot are orthologous. One explanation for this lack of support could be that the godwit and knot split a rather long time ago (app. 57 million years ago, Baker et al. 2007) and that the putative trans-species phylogenetic relationship among full length and 3bp deletion alleles has been lost though certain motifs remains.

Sites subject to positively selection is a well-known feature in the PBR of classical MHC alleles. We found between two and four such sites in godwit MHC alleles and interestingly some of these residues fell outside the PBR region inferred from chickens and humans (Wallny et al. 2006). Knots and gulls have a larger number of residues in exon 3 that are subject to positive selection than godwits (Cloutier et al. 2011; Buehler et al. 2013). The limited evidence for positive selection in the PBR of godwits is to a large extent due to the high rates of synonymous substitution in the PBR, particularly so for 3bp deletion alleles.

High rates of synonymous substitutions have been previously reported for MHC-I alleles among both passerine and non-passerine species (Westerdahl et al. 1999; Alcaide et al. 2013; Gonzalez-Quevedo et al. 2015). As in the studies of positive selection in the PBR of passerines, we have been including several MHC genes in the analyses, due to the difficulty of locus assignment, and this approach could also make it more difficult to find sites subject to positive selection.

Comparison of MHC polymorphism among three species within Charadriiformes showed that godwit MHC-I alleles exhibit overall intermediate levels of segregating sites, nucleotide diversity and evolutionary divergence compared to knots and gulls. Outside the order Charadriiformes, our data on godwit MHC polymorphism is similar to those described for other non-passerine species (Strandh et al. 2011; Alcaide et al. 2013). Nonetheless the godwit MHC polymorphism is still lower than that among passerines (Schut et al. 2011; Sepil et al. 2012; Gonzalez-Quevedo et al. 2015). In contrast to godwits, the high polymorphism in passerines may be associated with their shorter lifespan, rapid evolutionary rate and larger effective population sizes, factors that allow higher effectiveness of positive selection (Takahata 1990; Welch et al. 2008; Alcaide et al. 2013).

Recombination, gene conversion and point mutation play important roles in MHC gene evolution (Hess and Edwards 2002; Spurgin et al. 2011). Recombination events in putatively classical MHC genes have been reported in knots and gulls (Buehler et al. 2013; Cloutier et al. 2011) and also in species from the bird orders, Passeriformes, Galliformes and Falconiformes (Alcaide et al. 2009; Wutzler et al. 2012; Alcaide et al. 2013; Zeng et al. 2016).In the godwit we only found support for recombination in a small subset of the MHC alleles, *i.e.* a single recombination point for 3bp deletion alleles. However, recombination events are also evident from the multiple reticulate and parallel splits seen among godwit alleles in the network topology.

Despite the thorough sequencing of MHC alleles in a single godwit individual with several primer combinations and deep sequencing with MiSeq Illumina in 84 individuals we saw no evidence for godwits having non-classical MHC genes. Non-classical genes have been reported in both knots and gulls where both species have at least two non-classical genes

(Cloutier et al. 2011; Buehler et al. 2013). These non-classical genes can easily be seen in the neighbour network of both knots and gulls where they form distant clusters with high bootstrap support. In the godwit neighbour network, no such distant clusters can be seen. Regarding the number of MHC loci, godwits have one to four loci (2-7 alleles) per individual, whereas knots have up to six loci and gulls up to four loci (Cloutier et al. 2011; Buehler et al. 2013). However, when focusing on only putatively classical loci and taking into account the presence of 3bp deletion alleles, the MHC-I organisation in godwits is considerably more similar to knots than to gulls. For example, both godwits and knots have 0-3 alleles with 3bp deletion per individuals and 1-6 (2-5) full length alleles. The higher similarity between godwit and knot MHC is expected since the separation between gulls (Lari) and waders (Scolopaci), occurred much earlier (around late Cretaceous; app. 66 MYA) than the split between *Calidris* spp. and *Limosa* spp., which happened around late Paleocene (app. 57 MYA) (Baker et al. 2007).

Migration has been suggested to impose higher pathogen selection than a sedentary life-style and migration could possibly be a factor shaping and driving MHC diversity (Westerdahl 2007; Westerdahl et al. 2014). However, leaving the tropics to breed in temperate or arctic regions could also free hosts from pathogens hence migration would then result in a lower pathogen selection pattern for migratory birds than for birds that stay all year in the tropics. Perhaps, the highest difference in MHC diversity should be expected between sedentary birds in tropical Africa and in northern temperate regions. A recent study comparing several migratory and non-migratory passerine species showed a difference in the MHC diversity that coincided with migration, *i.e.* migratory species seemed to have a higher MHC diversity than sedentary European species (O'Connor et al. 2016). However, O'Connor et al. (2016) in particular reported that there is a strong phylogenetic signal in MHC diversity, so our hypothetical reasoning here on MHC diversity in migratory and non-migratory species is indeed preliminary and perhaps it is the phylogenetic past that to the largest degree explain the MHC diversity. The migratory behaviour in Charadriiformes seems to coincide with higher MHC diversity also in our study, though based on three species only, knots and godwits are both long-distance migrants while the gull is resident (Cloutier et al. 2011; Delany et al. 2009).

Even though godwits and knots have similar MHC diversity godwits still have MHC alleles with lower polymorphism and fewer sites subject to positive selection. One important difference between our study and the knot study, which might explain some differences regarding the polymorphism, is that we investigated only one subspecies whereas Buehler et al. (2013) investigated two sub-species (*C. c. islandica* and *C. c. rufa*) with different migratory strategies and pathogen exposure (Buehler and Piersma 2008). Regarding life-style and migratory strategies the Icelandic godwits investigated in the present study are more similar to *C. c. islandica* and hence the low polymorphism in these godwits could reflect their low exposure to pathogens resulting in a limited selection from pathogens. It would indeed be interesting to investigate the MHC diversity in additional subspecies of godwits, for example in the nominate (*L. l. limosa*), which is likely to be subjected to a different selection pressure from pathogens, possibly more diverged, than the Icelandic subspecies.

## Conclusions

Godwits seem to only have putatively classical MHC-I genes which is in contrast to two other Charadriiformes species, gulls and knots, that have both putatively classical and non-classical genes. Godwit MHC-I alleles have few sites subject to positive selection compared to gulls and knots and we believe that one explanatory factor is a limited selection from pathogens in godwits, particularly so for the Icelandic subspecies of black-tailed godwits that we have investigated in the present study.

# Appendix

**Table S1** - Primers used to cover most of Major Histocompatibility Complex (MHC) class I gene of Icelandic black-tailed godwits, *Limosa limosa islandica*. Star signal "*" stands for primers designed during this study and *T*a for annealing temperature. Partial (p) and full coverage of exons (Ex) were obtained by the use of different primer combinations. The differences in sequence length is because of a 3 base pair (bp) deletions in some exon 3 sequences.

| Primer name | Direction | Primer sequence 5´-3´ | *T*a (ºC) | Coverage | Sequence length (inner part) | References |
|---|---|---|---|---|---|---|
| *LiliM2F** | Forward | GGCCCCACTCCCTGCGTTAC | 65.5 | p Ex2 -Ex3 - p Ex4 | 733 – 736 bp | This study |
| *P126* | Reverse | AGTACCRGTGCCGBGTGGAGCA | 65.5 | | | Strandh et al. 2011 |
| *LiliM2F* | Forward | GGCCCCACTCCCTGCGTTAC | 65.5 | p Ex2 - p Ex3 | 476 – 479 bp | This study |
| *A23H8** | Reverse | GAGATACGTGAGCTACGGGC | 61.4 | | | |
| *A21P2* | Forward | TGGAGGACGGTAGCACCAG | 61 | p Ex3 | 174 – 177 bp | Strandh et al. 2011 |
| *A23H8* | Reverse | GAGATACGTGAGCTACGGGC | 61.4 | | | This study |
| *LiliM23F** | Forward | CAGGGCCCCACTCCCTGC | 65.1 | p Ex2 | 229 bp | This study |
| *LiliM2R** | Reverse | CCGCTACAACCAGAGCVGG | 62.4 | | | |
| *LiliM23F* | Forward | CAGGGCCCCACTCCCTGC | 65.1 | Ex2 - Ex3 - p Ex4 | 741 bp | This study |
| *P126* | Reverse | AGTACCRGTGCCGBGTGGAGCA | 65.5 | | | Strandh et al. 2011 |
| *LiliM3F** | Forward | TCGYGTTCCAGGGGCTCACA | 62.4 | p Ex3 | 244 – 247 bp | This study |
| *LiliM3R** | Reverse | GGCYGTGCTGGAGAGGAAA | 59.9 | | | |
| *LiliM3F2** | Forward | GGCTGTGASCTCCTGGAGGA | 63.5 | p Ex3 | 227 – 230 bp | This study |
| *LiliM3R* | Reverse | GGCYGTGCTGGAGAGGAAA | 59.9 | | | |

**Table S2 -** Inference of positive selection on 240-243 bp alleles of exon 3 (α2) of Icelandic black-tailed godwits (*Limosa limosa islandica*). Summary of the number of codon sites identified by the various positive and negative selection Maximum likelihood methods at the default significance values.

| Number of sites | Exon 3 alleles | SLAC (P<0.1) | FEL (P<0.1) | REL (P>50) | Integrative analysis |
|---|---|---|---|---|---|
| Positive selected | Combined | 3 | 7 | 6 | 4 |
| | Full length | 2 | 3 | 14 | 3 |
| | With 3 bp deletion | 0 | 0 | 13 | 2 |
| Negative selected | Combined | 9 | 13 | 20 | 11 |
| | Full length | 9 | 12 | 13 | 8 |
| | With 3 bp deletion | 1 | 7 | 1 | 2 |

**Table S3 –** Confirmed Major Histocompatibility Complex class I (MHC) alleles obtained by cloning and sequencing MHC from one Icelandic black-tailed godwit individual (*Limosa limosa islandica; Lili-UA*). Partial (p) and full coverage of exons 2, 3 and 4 were obtained by the use of different primer combinations. Displayed are the number of clones identified for each allele with the length of the sequences presented in the brackets. When several primer combinations were used a length distribution is reported.

| | cDNA | | | | gDNA | |
|---|---|---|---|---|---|---|
| Exon coverage per allele (length/N seq) | p Ex2-3-p Ex4 | p Ex2-p Ex3 | p Ex2 | p Ex3 | p Ex3 | Total |
| Lili-UA*01 | 20 (733 – 738) | 5 (476) | 4 (229) | 24 (174 – 244) | 3 (174) | 56 |
| Lili-UA*02 | 12 (733 – 738) | 0 | 0 | 2 (174) | 1 (174) | 15 |
| Lili-UA*03 | 4 (736 – 741) | 0 | 0 | 2 (247) | 0 | 6 |
| Lili-UA*04 | 1 (736) | 0 | 0 | 2 (177) | 5 (177) | 8 |
| Lili-UA*05 | 0 | 1 (476) | 0 | 8 (174 – 244) | 3 (174) | 12 |
| Lili-UA*06 | 0 | 0 | 0 | 0 | 2 (177) | 2 |
| Lili-UA*07 | 0 | 0 | 0 | 0 | 1 (177) | 1 |
| Total number of clones | 37 | 6 | 4 | 38 | 15 | **100** |

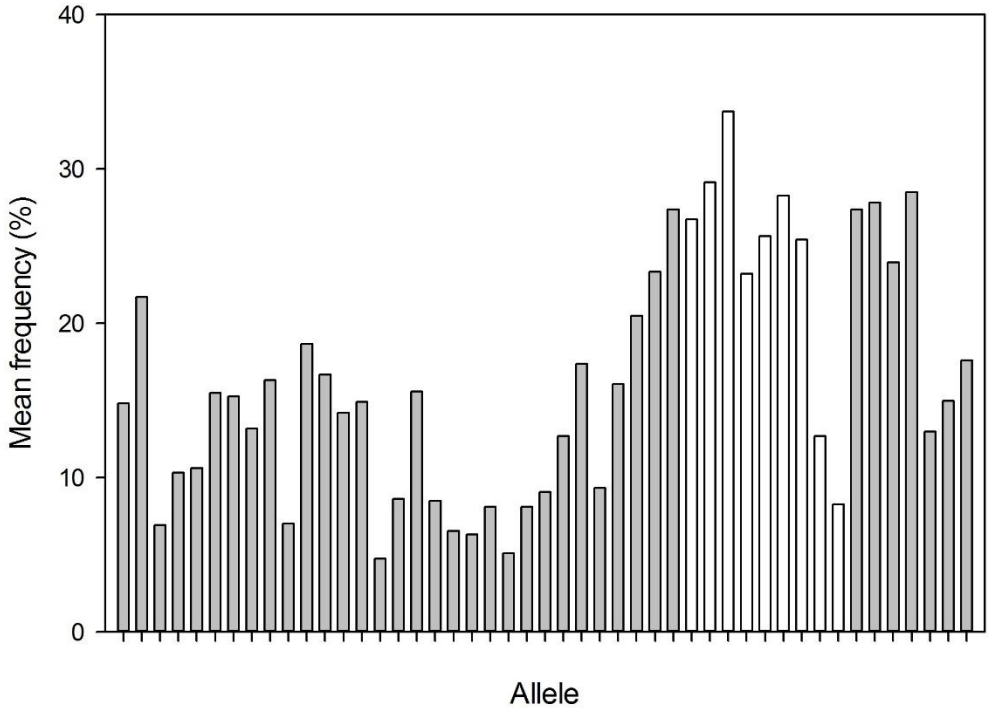



**Fig. S1 –** Frequency distribution of MHC-I exon 3 alleles from the 84 Icelandic black-tailed godwits (*Limosa limosa islandica*) individuals screened with Illumina MiSeq sequencing. Grey columns indicate full length alleles, while white columns indicate 3bp deletion alleles.

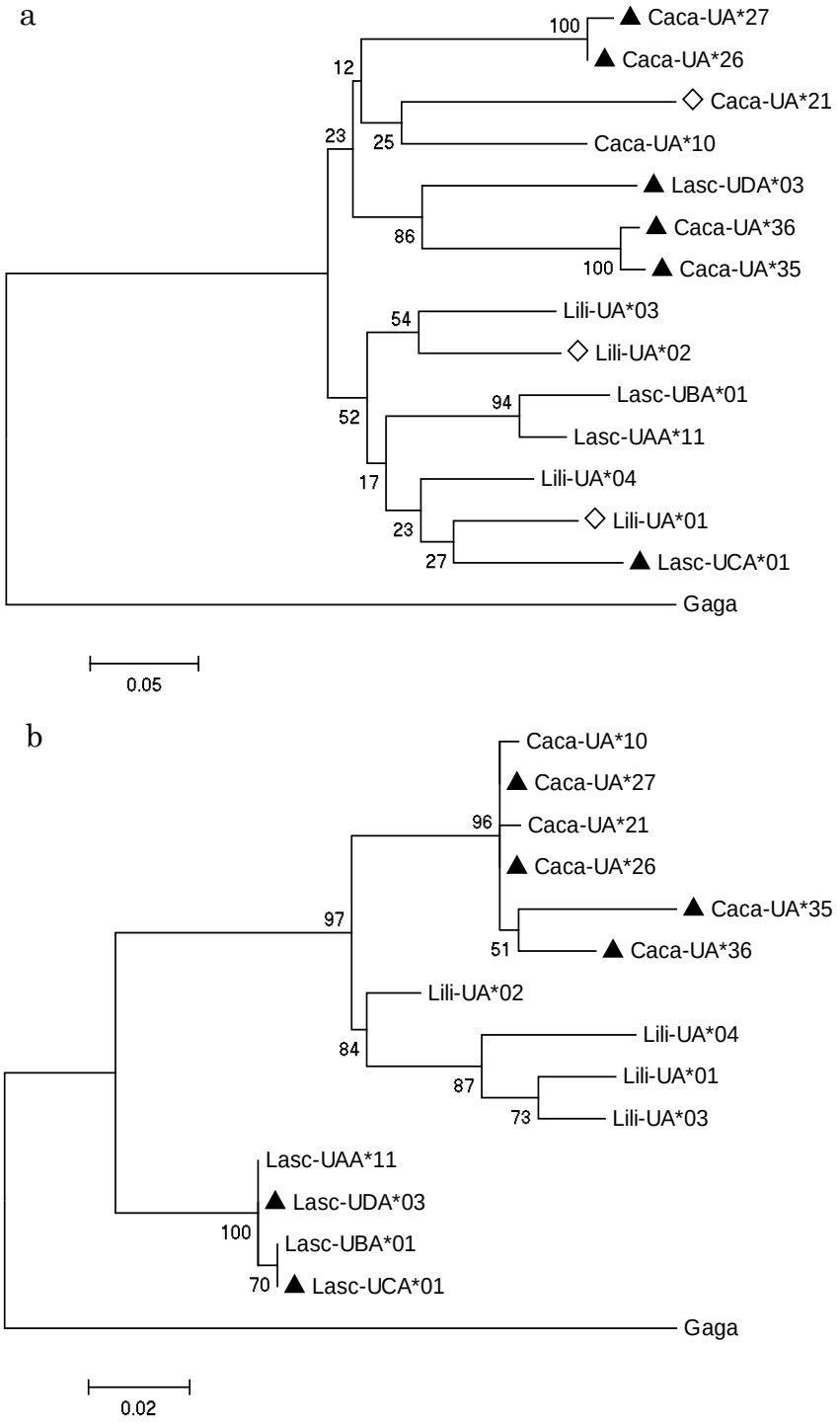



**Fig. S2 –** Nucleotide phylogenetic reconstruction of MHC-I from three Charadriiformes species; the Icelandic black-tailed godwit (Lili, *Limosa limosa islandica*), the red knot (Caca, *Calidris canutus*) and red-billed gull (Lasc, *Larus scopulinus*) using Maximum Likelihood with domestic chicken (Gaga, *Gallus gallus domesticus*) as an outgroup. Trees were done separately for (a) exon 2 and 3 holding the

PBR, and (b) for exon 4 encoding the structural part of MHC. Putatively classical alleles in the three species are: Lasc-UAA*11 (HM008713.1), Lasc-UBA*01 (HM008714.1), Caca-UA*10 (KC205115.1), Caca-UA*21 (KC205126.1) and Lili-UA*01 to Lili-UA*04. The remaining alleles are non-classical (marked with a black triangle): Lasc-UCA*01 (HM008715.1), Lasc-UDA*03 (HM008716.1), Caca-UA*26 (KC205131.1), Caca-UA*27 (KC205132.1), Caca-UA*35 (KC205140.1) and Caca-UA*36 (KC205141.1). On exon 3 Lili-UA*01, Lili-UA*02 and Caca-UA*21 have a 3bp deletion (marked with a white diamond), while Caca-UA*26 and Caca-UA*27 have a 12bp insertion. Numbers on branches indicates bootstrap values after 1000 repeats.

# Chapter III

## Is ecologic pathogen pressure a driving force of MHC-I diversity?


**“It takes all the running you can do, to keep in the same place”**

**as the Red Queen said to Alice in Wonderland**

**Carroll 1872**

# Abstract

Understanding how natural selection maintains adaptive variation in natural populations is fundamental in evolutionary ecology. Pathogens are unarguably among the strongest selective forces for maintaining genetic diversity in the Major Histocompatibility Complex (MHC), and MHC proteins play a crucial role in the recognition of pathogens. To shed some light upon the molecular genetic details of MHC genes subjected to natural selection we investigated the patterns of selection in MHC-I exon 3 in two closely-related subspecies of long-distance migratory Black-tailed godwits (*Limosa limosa*). Nominate godwits (*L. l. limosa*) are exposed to a higher risk of infection by pathogens (especially vector-borne) along their migration route compared to Icelandic godwits (*L. l. islandica*). We found that the loci number, allelic diversity and number of functionally different alleles per individual are different between the two subspecies. Nominate godwits (*L. l. limosa*) have more loci (2-4) and higher allelic diversity (4-7 alleles) and these patterns remain when measured as functional amino acid alleles. In addition, nominate godwits have almost the double number of sites subject to positive selection compared with Icelandic godwits. Overall the MHC-I polymorphism of both subspecies is similar, although the nominate tends to have more divergent private alleles. The higher MHC-I allelic diversity found for the nominate godwits can be explained by both, selection from pathogens and by neutral processes, the latter due to a larger population size. The two godwit subspecies separated recently (approximately at 11,000 Ma), our results seem to indicate that most MHC-I exon 3 differences are a reflection of parasite communities they encounter along the flyway. Nominate godwits are restricted to parasite-rich areas, which have led to a stronger diversifying selection of MHC genes and larger number of positively selected sites.

**Keywords** – Major histocompatibility complex, class I, black-tailed godwits, wader, pathogen pressure, habitat-related

# Introduction

The Major Histocompatibility Complex (MHC) encode highly polymorphic proteins of central importance in the immune system (Hess and Edwards 2002; Kaufman 2008; Murphy and Weaver 2017) and MHC diversity has therefore been the focus of intense research to understand details of molecular genetic diversity in the context of disease resistance, survival and fitness. Diseases are known to be important selective forces in the maintenance of MHC diversity within populations (Piertney and Oliver 2006; Spurgin and Richardson 2010), though less is known about differences in MHC diversity between populations, particularly so for populations that are known to differ in selection from pathogens. Populations that are subjected to stronger selection from pathogens are expected to have higher diversity than populations subjected to weaker selection. Prugnolle et al. (2005) found such pattern when studying human MHC diversity and adding distance to tropical Africa where the selection from pathogens is known to be most intense.

In birds it is possible to add an additional parameter to the study of pathogen selection and MHC diversity, namely migration. Characterization of MHC diversity in long-distance migrant birds can give a valuable contribution of how exposure to a broad scope of parasite faunas, shape and drive host MHC diversification and evolution, and how specific alleles confer selective and adaptive advantages to individuals in an ecological context. Bird species whose migratory strategies impose a differential pathogen exposure could become suitable models not only to disentangle the specific mechanistic processes behind MHC diversity, but also to gain a broader insight on how migrants cope with multiple parasite faunas across their flyways.

Pathogen pressure, which is known to vary both spatially (Salkeld et al. 2008; Yohannes et al. 2009) and seasonally (reviewed in Altizer et al. 2006), has been suggested to drive migration routes (Gill et al. 2009). It is hypothesized that many life-history features, such as migratory strategies and habitat choice of several migrant wader species, may have led to a differential ability to fight disease (Piersma 1997, 2003). Outside the breeding season and for many latitudinal migrant wader species, high arctic breeders are restricted to marine/coastal habitats, while temperate breeders use freshwater/inland habitats (Piersma

1997, 2003), which for example, leads to different exposure to vector-borne infections. A recent study by Clark et al. (2015), using a global dataset of avian malaria prevalence on migratory waders, provided further support for this hypothesis, showing that wader species that avoid the tropics and are restricted to marine habitats, have a lower risk of malaria infection. Low pathogen risk in Arctic and marine/coastal habitats may therefore spare waders from high investments in immune defence (Horrocks et al. 2011; Horrocks 2012; Sandström et al. 2014; Pardal et al. *submitted* a). Such habitat-use is a marked behaviour of two closely-related subspecies of Black-tailed godwits *Limosa limosa* (hereafter godwits), that are extremely dependent on wetland areas. One of the godwit subspecies, *Limosa limosa islandica* (hereafter Icelandic godwits), are subarctic breeders and wintering in south west Europe and they are restricted to brackish water coastal environments, while the other subspecies, *Limosa limosa limosa* (hereafter nominate godwits), uses inland freshwater habitats, breeding on temperate areas and wintering in the tropics (Gill et al. 2007; Delany et al. 2009). These closely related godwit subspecies are an excellent model to address how environmental exposure to pathogens, high in the nominate and low in the Icelandic, may have driven MHC loci variation in natural populations.

By their crucial role on encoding proteins that recognize foreign antigens, MHC genes are key elements for host-pathogen interactions and evolutionary adaptation (Sommer 2005; Spurgin and Richardson 2010). Other processes (*e.g.* mutation, recombination and duplication) are known to generate the high degree of MHC polymorphism (Edwards and Hedrick 1998; Martinsohn et al. 1999; Balakrishnan et al. 2010), but the prevailing evolutionary mechanism behind maintenance of polymorphism and allelic diversity for natural populations remains unclear (Bernatchez and Landry 2003). There is a general agreement for polymorphism to be maintained by balancing selection, and for pathogen-mediated selection to be a crucial driver (Sommer 2005; Piertney and Oliver 2006; Spurgin and Richardson 2010; Ejsmond et al. 2014). High variation at MHC loci is expected to increase T-cells repertoire and thus the chances of recognition of a specific antigenic peptide (Dyall et al. 2000). Heterozygous individuals at the MHC loci may provide an enhanced resistance to disease, which incurs in higher fitness (Doherty and Zinkernagel 1975; Sommer 2005; Milinski 2006; Spurgin and Richardson 2010), a strategy that is only beneficial in a

parasite-rich habitat context, due to the increased risk of developing autoimmune disorders (Ridgway et al. 1999).

In the present study we used high-throughput sequencing (MiSeq Illumina) in order to: (1) genotype MHC-I diversity of exon 3, (2) investigate genetic polymorphism, evidence of positive selection and antigen-binding properties, and (3) compare the MHC-I genetic organisation, polymorphism, positive selection and functional characteristics of the two godwit subspecies to address the impact of pathogen pressure modulation upon MHC genes.

# Material and methods

*Study subspecies and fieldwork*

The Black-tailed godwit is a long-distance migratory wader with a large geographical distribution, and within the western Palearctic only the nominate and Icelandic subspecies occur (Delany et al. 2009). Icelandic godwits breed in subarctic (Iceland) and nominate godwits breed in temperate latitudes (*e.g.* The Netherlands) (Jensen and Perennou 2007; Delany et al. 2009). Outside the breeding season, Icelandic godwits winter on brackish/coastal areas such as estuaries and large intertidal mudflats, along the Atlantic coast to Iberia and Morocco (Alves et al. 2010; Alves et al. 2013), while nominate godwits prefer inland freshwater habitats such as lake shores and irrigated rice-fields and move further south to west African areas (*e.g.* Senegal, Guinea-Bissau) (Gill et al. 2007; Hooijmeijer et al. 2013). During the breeding season of Icelandic godwits (May to July of 2011, 2012 and 2013) and nominate godwits (April–June 2014), chick-rearing adults were captured on two different study areas with the use of nest traps. Icelandic godwits were captured in the Southern lowlands of Iceland (64°1'46.14"N, 20°59'6.04"W), while nominate godwits were caught in Friesland, northern part of The Netherlands (53°10'19.79"N, 5°46'35.60"W). Captured birds were blood sampled from the leg or braquial vein (ca. 90 µl), ringed and released unharmed. Blood for genomic DNA (gDNA) was kept in 96% ethanol at -20ºC during fieldwork and then stored at -80ºC until laboratory analysis. In total, this study is based on

84 samples of gDNA (36 females and 49 males) from Icelandic godwits and 47 samples (30 females and 17 males) from nominate godwits collected in 2011-2014.

*Genomic DNA extraction and MiSeq Illumina sequencing on MHC-I exon 3*

Total genomic DNA was extracted by an adapted ammonium acetate protocol (Richardson et al. 2001). We used the high-throughput Illumina MiSeq sequencing for large scale subspecies gene amplification and genotyping of MHC-I α2 domain (exon 3), with primer pairs Lili M3F (5´-TCGYGTTCCAGGGGCTCACA–3´) and Lili M3R (5´-GGCYGTGCTGGAGAGGAAA–3´). Primers satisfactorily amplify a 247 bp of the 276 bp full exon 3 fragment and were especially designed for a MHC I characterization study of the target species (Pardal et al. *submitted* b). Each 25 µl Amplicon PCR reaction contained 12.5 µl 2X Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA) and 0.5 µM of each primer and 1 ng of gDNA template. The two step PCR profile was set to 25 cycles 98°C (10 s) and 72°C (15 s), ending with 72°C for 10 minutes. PCR products were checked on a 2% agarose gel and cleaned with Agencourt AMPure XP-PCR Purification kit (Beckman Coulter, Indianapolis, USA), by adding 20 µl AMPure XP beads to each reaction, washing with 75% ethanol and adding 43 µl of $ddH_2O$ for elution. After demultiplexing, recovery of individual amplicons was done by adding a unique combination of forward and reverse Illumina index to each sample by using Nextera XT v2 Index Kit (Illumina Inc., San Diego, CA, USA). A 25 µl PCR reactions volume were prepared containing 12.5 µl 2X Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 3 µl of each index primers and 2 µl of cleaned PCR amplicon product. The PCR profile was set to eight cycles at 98°C (10 s), 55°C (30 s) and 72°C (15 s), ending with 72°C for 5 minutes. PCR products were checked on a 2% agarose gel and cleaned with Agencourt AMPure XP-PCR Purification kit (Beckman Coulter, Indianapolis, USA) as mentioned above, except for the addition of 23 µl AMPure XP beads to each sample and 38 µl of $ddH_2O$ for elution. Cleaned PCR products were checked again on a 2% agarose gel and concentration was measured with a modified for a 96 well plate Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific/Invitrogen, Waltham, USA). Each plate was later equimolarly pooled, quantified with Qubit (Thermo Fisher Scientific, Waltham, USA) and run on Bioanalyzer DNA 2100 chip for quality and size

validation. When the desired concentrations were reached, pools were equimolar taken together to a final 4 nM library and sent for 300 bp paired-end Illumina MiSeq sequencing (Illumina Inc., San Diego, CA, USA) at the DNA sequencing facility Department of Biology, Faculty of Science, Lund University.

*Filtering MiSeq Illumina data*

Post-processing demultiplexing, clustering and filtering of next-generation sequencing data relied on Amplicon Sequencing Analysis Tools (AmpliSAT) (web server http://evobiolab.biol.amu.edu.pl/amplisat/; Sebastian et al. 2015). We used a four step procedure to cluster, filter and remove artefacts from our dataset, having in mind key assumptions from previously described methods (Galan et al. 2010; Lighten et al. 2014; Stutz and Bolnick 2014). Step 1) to allow detection of obvious reductions in read depths between amplicons and remove poor quality data that could introduce bias into the analysis a linear plot of amplicon read depths was produced. After analysis of individual amplicon data, the minimum required read depth was set to 4000 reads per amplicon. Step 2) based upon exploratory analysis we set to 25% the minimum frequency in respect to the dominant sequence threshold, meaning that sequences within a cluster that have ≥25% of the read depth compared to the dominant sequences were classified as "subdominants" and formed a new cluster. Step 3) since there is no information regarding the number of MHC-I loci for godwits for the establishment of a suitable per amplicon frequency (PAF), the threshold was determined by the best match between technical replicates (n=11 samples) in a similar fashion to Karlsson and Westerdahl (2013) and O´Connor et al. (2016). Optimal matches were obtained with a PAF of 3.4%. Sequences with a lower PAF were considered artefacts and removed. Lastly in step 4) only sequences differing 3bp from the expected length were kept (*i.e.* keeping sequences that were between 244 and 247 bp), while the remaining were discarded. A total of 1,620.634 reads remained for analysis after removal of reads with averages of Q≤30. For more details, see online resource.

Ultra-deep sequencing was achieved across samples and after filtering MiSeq data, Icelandic godwits had 11,322 ±335 ($\bar{X}$ ± SE) reads (Pardal et al. *submitted* b) and nominate had 12,008 ±211 ($\bar{X}$ ± SE) reads. Two samples from nominate godwits didn´t meet the amplicon depth

criteria (minimum of 4000 reads) for reliable genotyping and were excluded with the filtering (online resource Fig. S1).

## Data analysis

*Validation and identification of putative functional alleles*

MHC-I alleles found by Illumina sequencing were considered valid after complying all the filtering parameters described above, and were verified for typical antigen-presenting MHC class I features (*e.g.* no frameshift mutations, premature stop codons). Alleles were blasted in NCBI GenBank to confirm that they were MHC-I alleles and if previously described in any other avian species. Unique alleles were uploaded to the GenBank database and given species-specific names, following the Klein et al. (1990) nomenclature for naming MHC alleles (*Mhc*Lili-UA*xx). After manual alignment of alleles in BioEdit 7.2.5 Sequence Alignment Editor, sequences were translated to amino acid. All alleles were in reading-frame and had features suggesting that they were functional.

*Analysis of allelic polymorphism, recombination and positive selection in godwits*

Concerning the general analysis of the 47 alleles found for the Icelandic godwits, information regarding positive selection, recombination and polymorphism analysis was already available from Pardal et al. (submitted) (Table 2). Analysis was done considering only alleles that were found to be unique (*i.e.* private) for the Icelandic subspecies (n=24). For the nominate godwits, it was undertaken considering all alleles (n=96; with and without a 3bp deletion), for full length alleles (n=71), for alleles with a 3bp deletion (n=25), and finally, taking into account only private alleles (n=73).

On both subspecies, polymorphism analysis comprised estimation of polymorphic amino acid sites ($S_{aa}$), average nucleotide diversity ($\pi$), evolutionary divergence for nucleotide sequences ($d_{nt}$) and amino acid sequences ($d_{aa}$). For positive selection analysis, average rates of synonymous ($d_S$) and nonsynonymous ($d_N$) substitutions and rates of $d_N/d_S$ in the PBR and non-PBR regions were estimated. To determine evidence of selection in the

PBR and non-PBR, a codon-based Z test were calculated according to the Nei-Gojobori method with a Jukes-Cantor correction and a 1000 bootstrap replicates for variance estimation. Estimates of average evolutionary divergence of nucleotides were obtained using Kimura 2-parameter (K2P) model (Kimura 1980) with a discrete Gamma distribution (5 categories) and transition/transversion rate included, while for amino acid, a p-distance model with uniform rates among sites was used instead. Both models were run with gamma distribution (α=1) and 1000 bootstrap. All the above analysis was carried MEGA 7.0.14 (Kumar et al. 2016).

The PBR region was labelled in accordance with the previously documented PBR patterns from human and chicken MHC-I molecule (Wallny et al. 2006), that overlap with patterns of positive selection in other bird species (*e.g.* Alcaide et al. 2009; Cloutier et al. 2011; Buehler et al. 2013; Pardal et al. *submitted* b). The α2 (exon 3) PBR codons were the following 3, 5, 19, 21, 28, 48, 51, 52, 56, 58, 61, 62, 65, 66, 69, 73 and 77. Fixed-site model analysis for inference of positive selection were run on exon 3 alleles using 12 PBR positions (Fig. 1) excluding main chain-binding highly conserved sites (codons: 48, 51, 52, 65 and 77) (Bjorkman et al. 1987; Saper et al. 1991; Wallny et al. 2006). Before testing for evidence of positive selection, we explored the presence of recombination breakpoints using the genetic algorithm for recombination detection (GARD), available through the Datamonkey webserver (www.datamonkey.org; Pond et al. 2006; Delport et al. 2010). Analysis was done for combined alleles (with and without a 3bp deletion) (n=40), for full length alleles (n=40) and for alleles with a 3bp deletion (n=25). Inferred GARD trees were afterwards used to perform positive selection analysis with Maximum Likelihood (ML) methods. Random-sites models, like ML were also used to describe the overall variation among sites (Furlong and Yang 2008). Ultimately final positive selection results were obtained by combining the output of the following ML methods: single-likelihood ancestor counting (SLAC) at P=0.1, fixed effects likelihood (FEL) at P=0.1 and random effects likelihood (REL) with BF > 50 (online resource Table S1), all implemented on the Datamonkey webserver (Pond et al. 2006; Delport et al. 2010).

*Neighbour-network phylogenetic analysis*

Neighbour-net phylogenetic network on exon 3 alleles of nominate godwits were built separately and concatenated (Icelandic and nominate godwits) using SplitsTree v.4.14.4 (Huson and Bryant 2006) and based on the substitution model KP2. For the Icelandic godwit phylogenetic network was already available from Pardal et al. (submitted).

Best-fit nucleotide substitution models were chosen based on the highest Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) scores. Analysis was performed using JModel test 2.1.10 (Posada 2008) and MEGA 7.0.14. All phylogenetic networks used partial exon 3 sequences (240 and 243 bp) and the model was run with a transition/transversion (R) rate of 1.9421, probability of invariable sites of 0.4470 (p-inv), a gamma distribution shape parameter (α) of 0.4250. Phylogenetic networks have proved to be adequate for evolutionary representations of MHC genes, once they are based on the assumption of mutation and speciation events, besides taking into account gene loss, duplication and recombination events (Bryant and Moulton 2004).

# Results

*MHC class I alleles*

Illumina MiSeq was used to genotype MHC-I exon 3 in Icelandic (n=84 individuals; n=6 replicates; 11,322 ±335 reads) and nominate godwits (n=45 individuals; n=5 replicates; 12,008 ±211 reads) and the repeatability in genotyping across the 11 replicates was 100%. In total we verified 120 exon 3 sequences, covering 80 out of the 91 amino acids of the α2 domain, and these 120 nucleotide sequences translated into 110 amino acid sequences. Forty- seven MHC-I alleles were previously verified in the Icelandic godwits (Pardal et al. *submitted* b) and here 96 MHC-I alleles among the nominate godwits (Genbank accession numbers to be submitted). The alleles were of two different lengths, either 240 or 243 bp, and 25 alleles out of 96 alleles had a 3bp deletion at amino acid position 54, hereafter 3bp deletion alleles (Table 1; Fig. 1).

The 96 nominate MHC-I alleles translated into 89 amino acid sequences and the 47 Icelandic alleles into 40. The 71 full length alleles of nominate and the 38 of Icelandic, were

translated to 64 and 31 amino acid sequences, respectively. The 3bp alleles were more similar across the subspecies and an equivalent number of nucleotide and amino acid alleles were found (Table 1).

**Table 1 –** Comparative table of Major Histocompatibility Complex class I exon 3 alleles found respectively in the 47 and 84 individuals of nominate black-tailed (*Limosa limosa limosa*) and Icelandic (*L. l. islandica*) godwits*.* Table comprises the number of nucleotide alleles and equivalent translated amino acid alleles, separated according to allelic features (full length *vs*. 3bp deletion) and the overall and private alleles found for each subspecies.

| Number of alleles | Nominate godwits | Icelandic godwits |
|---|---|---|
| **All** | | |
| Nucleotide | 96 | 47 |
| Amino acid | 89 | 40 |
| Full length nucleotide | 71 | 38 |
| Full length amino acid | 64 | 31 |
| 3bp deletion nucleotide | 25 | 9 |
| 3bp deletion amino acid | 25 | 9 |
| **Private** | | |
| Nucleotide | 73 | 24 |
| Amino acid | 70 | 21 |
| Full length nucleotide | 56 | 23 |
| Full length amino acid | 53 | 20 |
| 3bp deletion nucleotide | 17 | 1 |
| 3bp deletion amino acid | 17 | 1 |

Functional characteristics of inter- and intra-domains and main-chain binding sites of the alleles were investigated exclusively among the nominate godwit alleles and they were highly conserved, as found previously for the Icelandic godwits (Pardal et al. *submitted* b). Exceptions were one inter and intra-domain position that was replaced by a glutamate (E; a polar amino acid) instead of glycine (G; a small amino acid) (position 17) on allele Lili-UA*112 (data not shown), a change that most likely have a substantial impact on allele function (Betts and Russell 2003). Also one main-chain binding position was found to be more variable (position 77) that the rest (data not shown). In this position, the tyrosine (Y) was replaced by histidine (H) in four alleles (Lili-UA*60, *66, *78, *80) and phenylalanine (F)

in nine alleles (Lili-UA*50, *58, *68, *88, *93, *98, *101, *106, *109), and this pattern was previously seen also among Icelandic godwit alleles (Pardal et al. *submitted* b). Interestingly, about half of the segregating amino acid sites (12 out of 23) were found outside the PBR sites (Fig. 1).

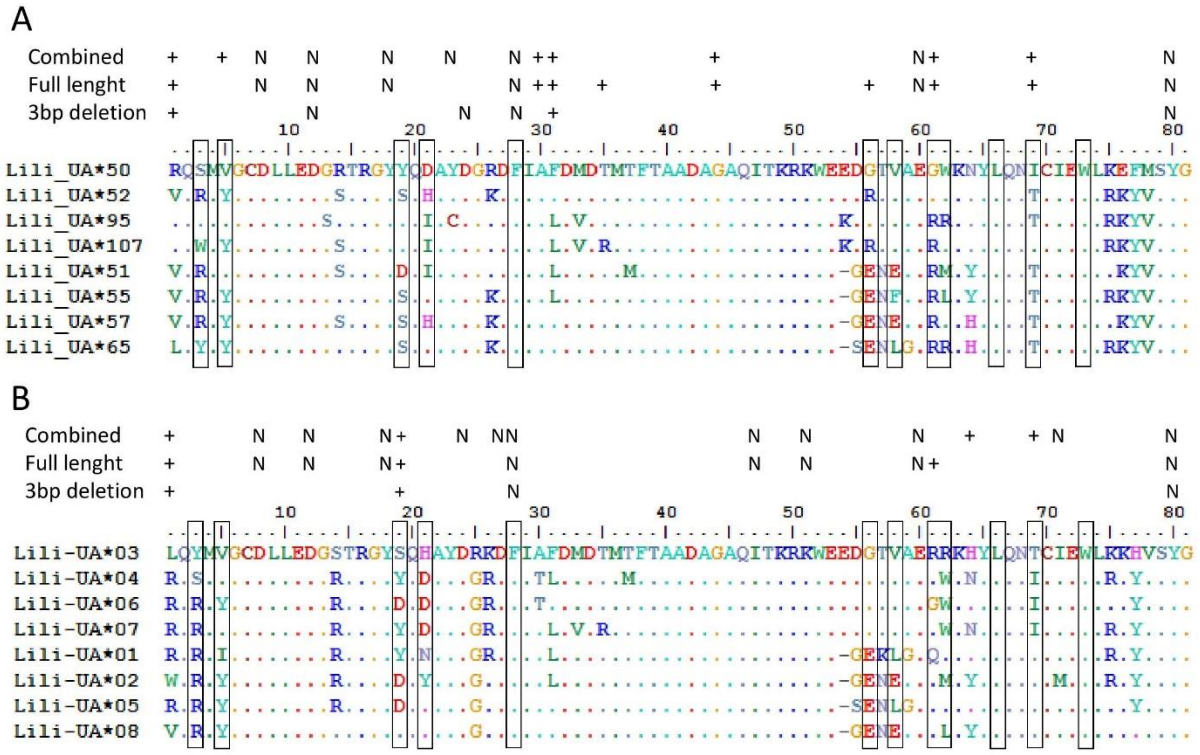



**Fig. 1 –** Alignment of (A) nominate black-tailed godwit Icelandic and (B) Icelandic black-tailed godwit MHC-I alleles covering 240-243 bp of the α2 region (exon 3) sequenced with MiSeq Illumina. Identity to Lili-UA*50 and Lili-UA*03 respectively are indicated by dots and dashes indicate gaps. Alleles Lili-UA*51, 55, 57, 65, 01, 02, 05 and 08 have a 3bp deletion at position 54. Boxes indicate PBR sites inferred from Wallny et al. (2006) excluding main-chain binding sites. Residues under positive selection (+), negative selection (N) were calculated using SLAC, FEL and REL methods (www.datamonkey.org; Pond et al. 2006; Delport et al. 2010).

*Exon 3 allelic diversity*

Nominate godwits have between four and seven different alleles per individual ($\bar{x}$ ±SE: 5.60 ±0.15) while Icelandic godwits have between two and seven different alleles ($\bar{x}$ ±SE: 4.86 ±0.12). Although there was a considerable overlap in number of alleles per individual between the subspecies, the nominate godwits had significantly more alleles per individual (Mann-Whitney U-test, Z= -3.28; *p*= 0.001). This patterns remains when the comparison is done on number of functional alleles (*i.e.* alleles with different amino acid sequences) (Mann-Whitney U-test, Z= -3.27; *p*= 0.001). Assuming heterozygosity, nominate godwits have two to four loci, while Icelandic have one to four MHC-I loci.

*Inference of positive selection, recombination and polymorphism in exon 3 alleles*

There are no overall differences between nominate or Icelandic godwits considering number of segregating amino acid sites, nucleotide diversity and divergence (nucleotides and amino acid distance; Table 2). When these analyses on polymorphism are done using only alleles that are private of each subspecies, then it is worth noting that the divergence drop among the Icelandic godwits, though there is still no statistically supported difference in divergence between subspecies (Table 3).

The dN/dS ratio was twice as high in the PBR relative to the non-PBR in exon 3 but the fixed-site model analysis showed no significant support for positive selection acting on the PBR of exon 3 in either nominate or Icelandic godwits (data not shown). The same pattern was found for private alleles, in which there were no significant differences between subspecies.

**Table 2 –** Measures of polymorphism and evolutionary divergence of the Major Histocompatibility Complex class I exon 3 alleles in the nominate black-tailed godwit (*Limosa limosa limosa*) and Icelandic godwits (*L. l. islandica*). Analysis included all alleles found on each subspecies, and both full length (243 bp) and 3bp deletion alleles (240 bp). The PBR is defined as 12 amino acids excluding main chain binding sites (see text for details). Table shows the number of alleles ($N_a$), estimates of non-synonymous $d_N$ ±SE and synonymous $d_S$ ±SE substitution rates, codon-based ratio between $d_N/d_S$, the overall number of polymorphic amino acid sites ($S_{aa}$) and average nucleotide diversity ($\pi$). Evolutionary divergence averages for nucleotides ($d_{nt}$) was estimated using the Kimura 2-parameter model and with p-distance model for amino acid distance ($d_{aa}$).

| | **Nominate godwits** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **$N_a$** | **dN±SE** | **dS±SE** | **$d_N/d_S$** | **$S_{aa}$** | **π** | **$d_{nt}$±SE** | **$d_{aa}$±SE** |
| **All** | | | | | | | | |
| PBR | 96 | 0.347±0.110 | 0.330±0.159 | 1.052 | 10 | 0.270 | 0.582±0.296 | 0.428±0.072 |
| Non-PBR | 96 | 0.047±0.010 | 0.082±0.023 | 0.573 | 34 | 0.045 | 0.049±0.017 | 0.101±0.020 |
| **Full length** | | | | | | | | |
| PBR | 71 | 0.252±0.089 | 0.211±0.149 | 1.194 | 10 | 0.229 | 0.440±0.264 | 0.331±0.068 |
| Non-PBR | 71 | 0.040±0.009 | 0.054±0.018 | 0.741 | 30 | 0.043 | 0.047±0.016 | 0.086±0.018 |
| **3bp deletion** | | | | | | | | |
| PBR | 25 | 0.294±0.111 | 0.338±0.158 | 0.870 | 8 | 0.254 | 0.499±0.285 | 0.339±0.089 |
| Non-PBR | 25 | 0.038±0.011 | 0.071±0.024 | 0.535 | 18 | 0.039 | 0.043±0.020 | 0.080±0.021 |
| | **Icelandic godwits** | | | | | | | |
| **All** | | | | | | | | |
| PBR | 47 | 0.320±0.106 | 0.317±0.158 | 1.009 | 10 | 0.255 | 0.560±0.285 | 0.394±0.068 |
| Non-PBR | 47 | 0.045±0.010 | 0.083±0.026 | 0.542 | 27 | 0.041 | 0.044±0.016 | 0.097±0.019 |
| **Full length** | | | | | | | | |
| PBR | 38 | 0.242±0.091 | 0.225±0.150 | 1.076 | 10 | 0.220 | 0.438±0.245 | 0.307±0.065 |
| Non-PBR | 38 | 0.039±0.009 | 0.060±0.022 | 0.650 | 23 | 0.039 | 0.043±0.016 | 0.085±0.018 |
| **3bp deletion** | | | | | | | | |
| PBR | 9 | 0.302±0.107 | 0.394±0.179 | 0.766 | 8 | 0.375 | 0.473±0.300 | 0.394±0.088 |
| Non-PBR | 9 | 0.041±0.012 | 0.078±0.027 | 0.526 | 14 | 0.089 | 0.040±0.019 | 0.093±0.023 |

**Table 3 –** Measures of polymorphism and evolutionary divergence of Major Histocompatibility Complex class I exon 3 alleles in the nominate black-tailed godwit (*Limosa limosa limosa*) and Icelandic godwits (*L. l. islandica*). Analysis included alleles found separately and exclusive for each subspecies as well as both full length (243 bp) and 3bp deletion alleles (240 bp). The PBR is defined as 12 amino acids excluding main chain binding sites (see text for details). Table shows the number of alleles ($N_a$), estimates of non-synonymous $d_N$ ±SE and synonymous $d_S$ ±SE substitution rates, codon-based ratio between $d_N/d_S$, the overall number of polymorphic amino acid sites ($S_{aa}$) and average nucleotide diversity (π). Evolutionary divergence averages for nucleotides ($d_{nt}$) was estimated using the Kimura 2-parameter model and with p-distance model for amino acid distance ($d_{aa}$).

| | **Nominate godwits** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **$N_a$** | **dN±SE** | **dS±SE** | **$d_N/d_S$** | **$S_{aa}$** | **π** | **$d_{nt}$±SE** | **$d_{aa}$±SE** |
| **All** | | | | | | | | |
| PBR | 73 | 0.335±0.107 | 0.319±0.155 | 1.050 | 9 | 0.260 | 0.544±0.303 | 0.418±0.075 |
| Non-PBR | 73 | 0.047±0.010 | 0.077±0.024 | 0.610 | 31 | 0.045 | 0.050±0.018 | 0.100±0.020 |
| | **Icelandic godwits** | | | | | | | |
| **All** | | | | | | | | |
| PBR | 24 | 0.235±0.095 | 0.243±0.155 | 0.967 | 9 | 0.204 | 0.382±0.232 | 0.297±0.069 |
| Non-PBR | 24 | 0.039±0.008 | 0.059±0.024 | 0.661 | 22 | 0.038 | 0.041±0.017 | 0.086±0.018 |

Positive selection estimation by ML methods on alleles of the nominate subspecies identified in total nine different sites subjected to positive selection in exon 3 (Fig. 1). The combined random alleles analysis found seven sites (positions 1, 5, 30, 31, 44, 61 and 69), while the analysis run on random full length alleles, identified eight sites (positions 1, 30, 31, 35, 44, 56, 61 and 69), in which six positions overlap with the combined analysis (positions 1, 30, 31, 44, 61 and 69). The ML analysis on the 3bp deletion alleles only identified two positively selected sites (position 1 and 31), both being already recognized by the combined and full length analysis. The same analysis done for the Icelandic godwits found in total only five sites subjected to positive selection (Pardal et al. *submitted* b) (Fig. 1). From all the positively selected positions identified by ML methods, only positions 5, 19, 56, 61, and 69 overlap with the PBR sites inferred by Wallny et al. (2006), while the rest were detected outside.

Recombination was investigated separately for the full length and 3bp deletion alleles of nominate godwits. There were no recombination points in the full length alleles, but GARD predicted one recombination point at amino acid position 37 in the twenty-five 3bp deletion alleles (data not shown). The results are similar to the ones found in Icelandic godwits, in which recombination points were only detected for 3bp deletion alleles at amino acid position 28 (Pardal et al. *submitted* b).

*Phylogenetic analysis*

Phylogenetic neighbour network trees built on MHC-I exon 3 nucleotide alleles showed a clear separation of full length and 3bp deletion alleles, both for nominate and Icelandic godwits (bootstrap of 70.2 for nominate and 61.3 for Icelandic; Fig. 2). A network tree built on MHC-I exon 3 from both subspecies, showed the same pattern (Fig. 3). The reticulation of the branches is indicative of conflicting signals that can either arise from recombination and/or gene conversion.

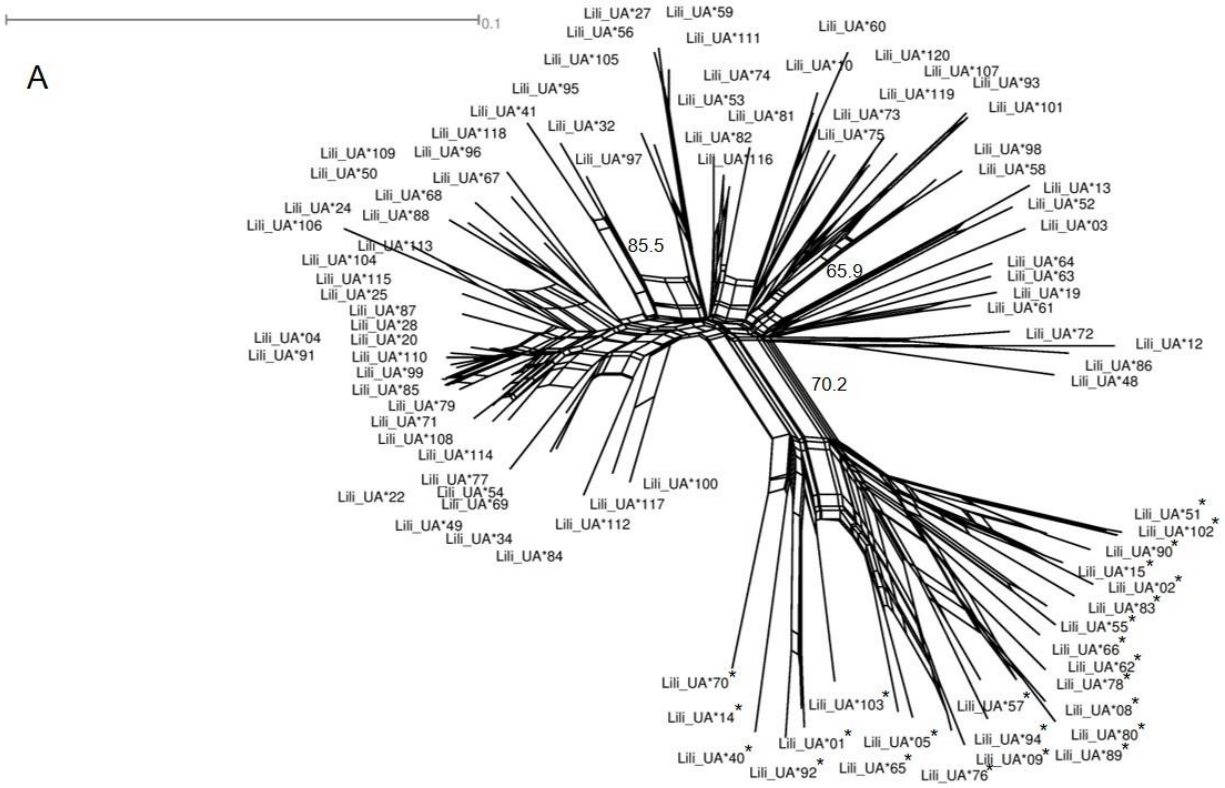

A
0.1
Lili_UA*27
Lili_UA*59
Lili_UA*56
Lili_UA*111
Lili_UA*60
Lili_UA*105
Lili_UA*74
Lili_UA*120
Lili_UA*107
Lili_UA*93
Lili_UA*95
Lili_UA*53
Lili_UA*119
Lili_UA*41
Lili_UA*81
Lili_UA*73
Lili_UA*101
Lili_UA*118
Lili_UA*32
Lili_UA*82
Lili_UA*75
Lili_UA*109
Lili_UA*96
Lili_UA*97
Lili_UA*116
Lili_UA*98
Lili_UA*50
Lili_UA*67
Lili_UA*58
Lili_UA*68
Lili_UA*13
Lili_UA*24
Lili_UA*88
Lili_UA*52
Lili_UA*106
Lili_UA*03
85.5
Lili_UA*113
65.9
Lili_UA*104
Lili_UA*64
Lili_UA*115
Lili_UA*63
Lili_UA*25
Lili_UA*19
Lili_UA*87
Lili_UA*61
Lili_UA*28
Lili_UA*04
Lili_UA*20
Lili_UA*72
Lili_UA*12
Lili_UA*91
Lili_UA*110
Lili_UA*86
Lili_UA*99
Lili_UA*48
Lili_UA*85
70.2
Lili_UA*79
Lili_UA*71
Lili_UA*108
Lili_UA*114
Lili_UA*77
Lili_UA*100
Lili_UA*22
Lili_UA*54
Lili_UA*69
Lili_UA*117
Lili_UA*49
Lili_UA*112
Lili_UA*34
Lili_UA*84
Lili_UA*51*
Lili_UA*102*
Lili_UA*90*
Lili_UA*15*
Lili_UA*02*
Lili_UA*83*
Lili_UA*55*
Lili_UA*66*
Lili_UA*62*
Lili_UA*78*
Lili_UA*70*
Lili_UA*08*
Lili_UA*14*
Lili_UA*103*
Lili_UA*57*
Lili_UA*80*
Lili_UA*01*
Lili_UA*05*
Lili_UA*94*
Lili_UA*40*
Lili_UA*09*
Lili_UA*89*
Lili_UA*92*
Lili_UA*65*
Lili_UA*76*
B
0.01
Lili-UA*27
Lili-UA*21
Lili-UA*10
Lili-UA*29
Lili-UA*32
Lili-UA*11
Lili-UA*12
Lili-UA*16
Lili-UA*44
Lili-UA*39
Lili-UA*18
Lili-UA*33
Lili-UA*24
Lili-UA*06
Lili-UA*34
Lili-UA*19
Lili-UA*38
22.3
Lili-UA*04
Lili-UA*47
52.2
Lili-UA*20
Lili-UA*13
Lili-UA*28, Lili-UA*30
Lili-UA*03
61.3
Lili-UA*42
Lili-UA*14*
Lili-UA*22
Lili-UA*26
45.3
49
Lili-UA*25
Lili-UA*31
Lili-UA*09*
Lili-UA*43
Lili-UA*35
100
Lili-UA*08*
Lili-UA*46
Lili-UA*17*
Lili-UA*37
Lili-UA*07
Lili-UA*15*
Lili-UA*41
Lili-UA*02*
Lili-UA*36
Lili-UA*23
Lili-UA*01*
Lili-UA*05*
Lili-UA*45
Lili-UA*40*

**Fig. 2 –** Neighbour-net phylogenetic network of MHC-I exon 3 alleles from: (A) nominate black-tailed godwits (*Limosa limosa limosa*), (B) Icelandic black-tailed godwits (*Limosa limosa islandica*). The represented 96 and 47 alleles from nominate and Icelandic godwits, respectively, are either full (243 bp) and partial (240 bp) lengths. Alleles with a 3bp deletion are indicated by a star "*". Bootstrap values after 1000 repeats for main splits are represented. Note the different range scales.

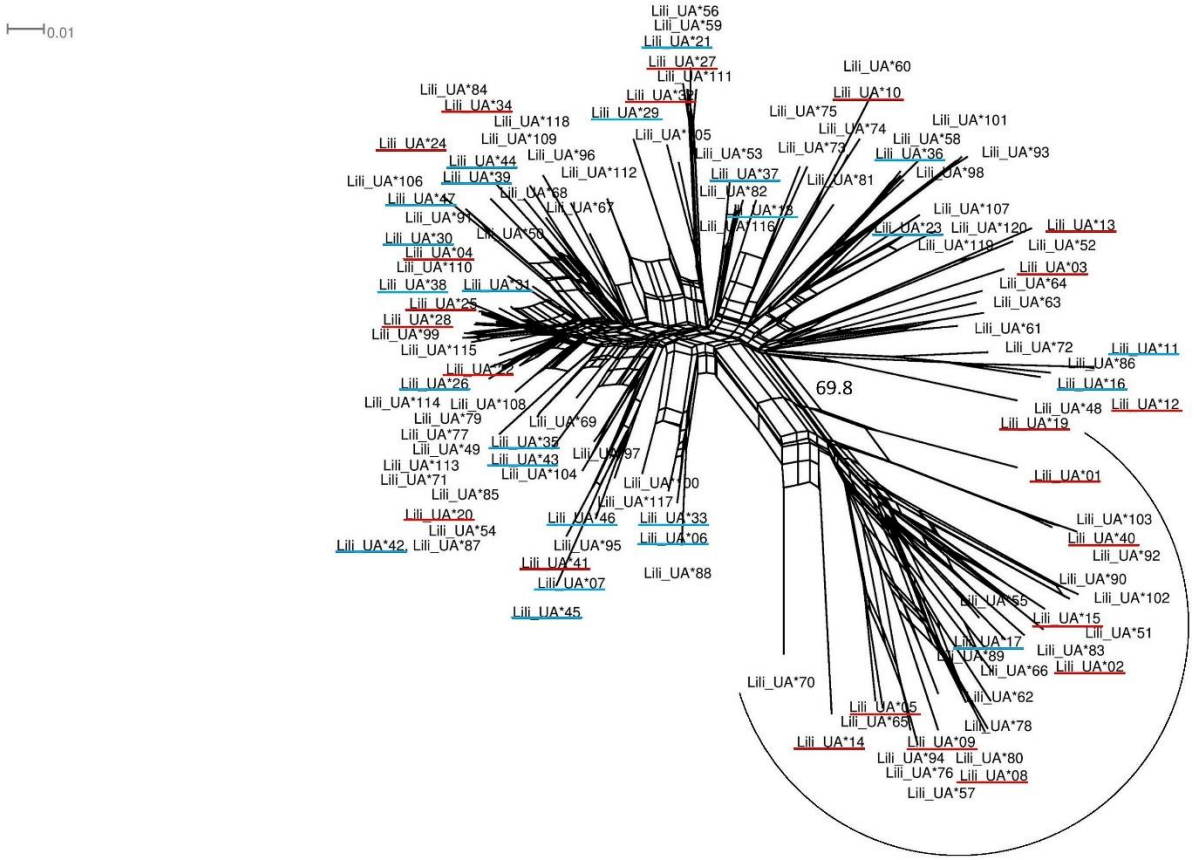



**Fig. 3 –** Neighbour-net phylogenetic network of MHC-I exon 3 concatenated subspecies alleles. Alleles underlined in blue are exclusive of Icelandic godwits (*Limosa limosa islandica*), in red are shared by both subspecies and with no underline are exclusive from nominate godwits (*Limosa limosa limosa*). The semi-circle contained cluster indicate alleles with a 3bp deletion. Network represents the 96 and 47 alleles from nominate and Icelandic godwits with either full (243 bp) or partial (240 bp) lengths. Bootstrap values after 1000 repeats for main splits are represented.

# Discussion

The migratory strategies and habitat occupancy differ between the two closely-related subspecies of black-tailed godwits, the nominate and the Icelandic (Delany et al. 2009), and as a consequence we hypothesized that there are differences in pathogen selective pressures that generate differences in the MHC polymorphism in these subspecies. The Icelandic godwits are restricted to relative parasite-free areas, especially vector-borne, compared to the nominate godwit. Pathogens play a prominent role upon natural selection and evolution is likely to have shaped the MHC genes in the subspecies according to the disparate disease landscapes they inhabit. It is therefore likely that the two diversified parasite communities encountered by the two godwit subspecies have led to differences in MHC diversity.

In this study, by investigating the patterns of selection in MHC-I exon 3, we found that the loci number, number of nucleotide alleles and number of functionally different amino acid alleles per individual was significantly higher for the nominate compared with the Icelandic godwits. Likewise, positive selection estimation was also higher for the nominate godwits, than for the Icelandic. Overall the MHC-I polymorphism of both subspecies is similar but, and though not statistically supported, the nominate private alleles are more divergent at the nucleotide and amino acid level, meaning that MHC-I alleles have a larger percentage of differences (*i.e.* the mutational substitutions). We also identified 96 different alleles for nominate godwits, while for the Icelandic godwits a previous study from Pardal et al. (submitted) reported 47 alleles. In total, from the 120 alleles described, both subspecies share 23 alleles, whereas 73 and 24 alleles were found to be private for nominate and Icelandic godwits, respectively. By comparison, in about half of the nominate individuals used in this study, we found almost the double of alleles has those described for the Icelandic godwits. Like in the Icelandic godwits and despite deep sequencing, we found no evidence of non-functional or putatively non-classical genes among nominate godwits (Pardal et al. *submitted* b), though at least two non-classical ones were previously reported on a closest *Scolopacidae* species, the red knot (hereafter knots, *Calidris canutus*) (Buehler et al. 2013).

Understanding patterns of selection in natural population is a difficult task since differences in the heterozygosity and diversity of several genes can also be attributed to neutral forces that do not result from selection, from *e.g.* parasites, but are instead driven by other mechanisms such as population demography and dispersal patters (Hughes and Yeager 1998; Sommer 2005). The godwit subspecies differ significantly in population size, with the nominate being estimated at around 170,000 individuals, while the Icelandic has approximately 47,000 individuals (Delany et al. 2009). These differences can thus reduce the overall genetic diversity of the Icelandic subspecies. In fact, a recent study by Trimbos et al. (2014) showed (though not statistically supported) that Icelandic godwits have a lower absolute number of alleles and allelic richness (per sampling location) of nuclear and mitochondrial genes, than nominate godwits. Therefore, the higher MHC-I allelic diversity found for the nominate godwits, may not only be attributed to diversifying selection from pathogens, but could also be due to neutral processes.

Trimbos et al. (2014) showed that the nominate and Icelandic subspecies separated fairly recently on an evolutionary time-scale (after the Pleistocene, app. 11,000 Ma) and therefore it is not surprising that the overall comparison of MHC allele polymorphism between the two subspecies gave similar values when it comes to number of segregating sites, nucleotide diversity and evolutionary divergence. Another possible explanation for the polymorphism similarities, could be due to sample size differences between subspecies that might be concealing patterns. The number of Icelandic individuals used in this study represent almost the double of nominate individuals, which might lead to polymorphism underestimates for the nominate subspecies and counteract for more obvious diversification patterns between the two. Differences between the subspecies, become more evident when comparing the polymorphism of private alleles and when taking into account the number of alleles that were translated into unique amino acid sequences. Across all the alleles found for the nominate, irrespective of being private or not, the number of nucleotide alleles that are translated into different amino acid sequences are always proportionally higher for the nominate species.

On subspecies neighbour networks, the 3bp deletion alleles form distinctive clusters even when the network was built independently of godwit subspecies (*i.e.* at the species

level). Alleles with deletions, were also found in knots and described has putatively functional (Buehler et al. 2013). Like for both subspecies of godwits, in knots 3bp deletion alleles cluster together, but despite the resemblance in arrangement, previous data does not support that these genes might be orthologous (Pardal et al. *submitted* b). As for both subspecies of godwits, despite the similar arrangement of their respective networks, it is important to underline that the allelic distances are considerably different between the two subspecies. For the nominate godwits, nucleotide allelic distances are far higher than the ones found for Icelandic godwits, again supporting that nominate alleles are more different from one another.

The PBR of MHC proteins is crucial for antigen binding and the PBR sites are subject to balancing selection. Balancing selection can be investigated by studying positive selection, hence the rates of non-synonymous substitutions and synonymous substitutions (Schmid-Hempel 2011). Despite only detected by random-site models, overall nominate godwit alleles had a higher number of sites subjected to positive selection (9 sites) when compared to the Icelandic godwits (5 sites). The number of positively selected sites are also comparable with the seven (on average) found across exon 3 MHC-I alleles of Charadriiformes (Buehler et al. 2013; Cloutier et al. 2011). Most of the positively selected sites in godwits fell outside the PBR region inferred from chickens and humans by Wallny et al. (2006), which could indicate that at least some of the sites might not be necessarily the same across species. For both godwit subspecies, most of the positively selected sites were found among full length and combined alleles, while analysis run only on 3bp deletion alleles added no new position to the previously found. Overall our data seems to indicate that positive selection is stronger on full length alleles, rather than for 3bp deletion alleles for both godwits.

We found that both synonymous and nonsynonymous substitution rates were higher across the PBR regions of nominate godwit alleles, being most likely the factor limiting the evidence for positive selection by fixed-site models in the PBR of nominate godwit MHC-I alleles. The high rates of synonymous substitutions were also seen among the Icelandic alleles (Pardal et al. *submitted* b). These patterns of substitution may be the result of two different factors, 1) the inclusion of several non-homologous loci into the analysis due to the impossibility of locus assignment with our data, and/or 2) an indication of gene conversion,

which is also a known mechanism to generate MHC variation (Hughes and Nei 1988; Ohta 1995; Chen et al. 2007). Despite being difficult to detect gene conversion in natural populations, this mechanism was already documented in birds (Wittzell et al. 1999; Burri et al. 2010; Spurgin et al. 2011). Recombination is another important mechanism in MHC evolution (Hess and Edwards 2002; Spurgin et al. 2011). Recombination break-points were detected among the 3bp deletion alleles of nominate godwits, similarly to what was found for the Icelandic godwits (Pardal et al. *submitted* b) and also in other Charadriiformes members (Cloutier et al. 2011; Buehler et al. 2013).

Despite that both godwit subspecies share a similar MHC polymorphism, our data of positive selection supports that nominate godwits are subjected to stronger selection from parasites. Habitat and spatial pathogen-pressure differences between the two subspecies are likely responsible for the different patterns of MHC differentiation that not only affect adaptive immunity, but also innate immunity (Pardal et al. *submitted* a). Population differences in adaptive immunity (MHC) have been reported previously in other bird species as well as in fishes (Miller et al. 2001; Wegner et al. 2003; Collin et al. 2013). Therefore, it seems that not only migration, but habitat-related differences on pathogen pressure can impose a higher selection at the MHC level.

The lack of comparative MHC characterization studies between migrant, partial migrants and sedentary species (or populations) with differential exposure to pathogen-pressure, become essential when addressing mechanistic processes behind MHC evolution and how specific alleles confer adaptive advantages to individuals on an ecological context. For the conservation point of view, this information becomes vital giving the higher susceptibility of long-distance migrants to the deleterious effects of genetic diversity loss of several genes (including MHC) through population reductions (Dlugosch and Parker 2008; Spurgin et al. 2011). Many species of long-distance migratory species are in sharp decline due to habitat loss, hunting and climate change (Sanderson et al. 2006). Our data in parallel with the one found by Trimbos et al. (2014) are indicative that Icelandic godwits may be more vulnerable to epizootic disease strikes due to their overall lower genetic diversity (neutral and MHC genes).

# Appendix

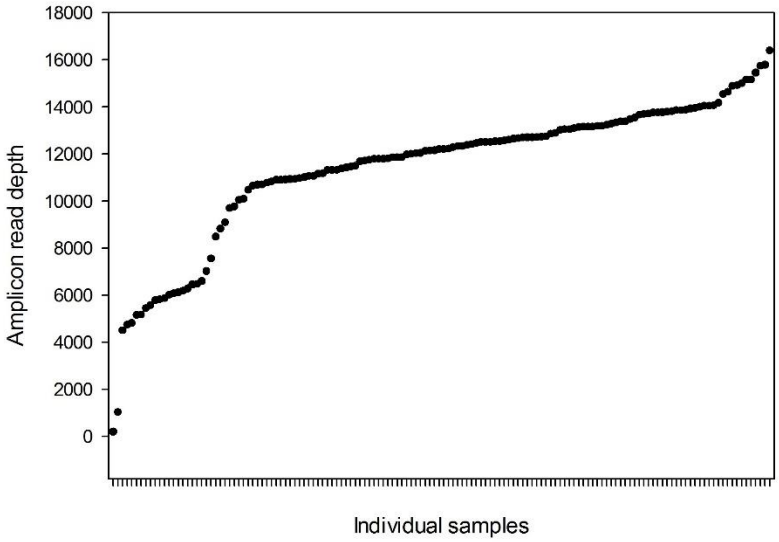



**Fig. S1** – Amplicon read depth plot. Individual samples below the 4000 read threshold belong to SP166 and SP175, both from nominate godwits (*Limosa limosa limosa*).

**Table S1** – Inference of positive selection on 240-243 bp alleles of exon 3 (α2) of nominate black-tailed godwits (*Limosa limosa limosa*). Summary of the number of codon sites identified by the various positive and negative selection Maximum Likelihood methods at the default significance values.

| Number of sites | Exon 3 alleles | SLAC (P<0.1) | FEL (P<0.1) | REL (P>50) | Integrative analysis |
|---|---|---|---|---|---|
| Positive selected | Combined | 7 | 11 | 14 | 7 |
| | Full length | 8 | 9 | 17 | 8 |
| | With 3 bp deletion | 1 | 4 | 8 | 2 |
| Negative selected | Combined | 8 | 11 | 17 | 7 |
| | Full length | 6 | 12 | 11 | 6 |
| | With 3 bp deletion | 4 | 8 | 7 | 4 |

# Chapter IV

## Dressed to impress: is breeding plumage an honest signal of innate immunity?


**"When the sexes differ in beauty, in the power of singing, or in producing what I have called instrumental music, it is almost invariably the male which excels the female."**

**Charles Darwin**

# Abstract

Parasites have long impacted several aspects of host fitness, but they are also known to affect sexual behaviour by mediating mate choice. Based on the hypothesis that sexual signalling is affected by parasites, sexual characters can be used as honest signals of the individual quality and immune responsiveness. Many studies have already assessed trade-offs between adaptive immunity and sexual traits, but few have tested the same assumptions for innate immunity in free-living migratory animals. Melanins, one of the most common animal colour pigments used in social communication are immunosuppressive and constitutive innate immunity used to control multiple pathogens in periods of both time and resource constraints, can have an adaptive function during the strenuous work of migration given their effect on oxidative stress and energy homeostasis. In this study we used Black-tailed godwits, *Limosa limosa*, a long distance migratory wader displaying melanin-based secondary sexual characters, and showed that melanin-based plumage features of male and female godwits are indeed linked to soluble, but not cellular, parameters of innate and adaptive immunity and are also condition-dependent. The honesty of the signal was not similar between the sexes: males exhibited a larger colour extent, and had higher circulating levels of haptoglobin and natural antibodies; whilst for females a negative relationship between colour extent and haemolysis capacity was found. Colour intensity was the only condition-dependent plumage feature, being negatively associated with darker coloured males, but not influencing female plumage. Sex differences in the honesty of the signal are likely driven by sexual roles and sex-specific energetic demands. Overall our results indicate that by choosing plumage exuberant males, female godwits are not only selecting partners who proved to be more capable of investing energy on both plumage and migration, but may be also less vulnerable to novel infections.

**Keywords:** Black-tailed godwit, sexual secondary characters, wild birds, condition dependent, baseline immunity, waders, migration

# Introduction

Sexual selection is a major evolutionary force leading to the evolution of some extreme ornamentation and colouration, often used in elaborated courtship behaviours across the animal kingdom (Andersson 1994). In recent years, it has been hypothesized that secondary sexual characters (SSC) are honest signals of individual quality, being directly linked to immune responsiveness and the ability to fight infection (Hamilton and Zuk 1982; Folstad and Karter 1992; Lozano 1994; Alonso-Alvarez et al. 2007). Based on the principle that immune response is energetic and resource costly, but at the same time an essential self-maintenance physiological component, trade-offs must be in operation between competing life-history traits, such as immune capacity and reproductive effort and investment (Sheldon and Verhulst 1996; Zuk and Stoehr 2002). Sexual signalling may be important if indeed indicates mate quality, which is assured through the costs as well as the fitness benefits of producing and maintaining SSC (*e.g.* Alonso-Alvarez et al. 2007). In addition it explains the female preference for males that exhibit pronounced sexual characters (Grafen 1990). Several studies found a direct link between immune capacity (in some cases also condition dependent) and male SSC in birds, such as the long tails of swallows (Saino et al. 1997), size of tail patch of greenfinches (Lindström and Lundström 2000), song complexity of passerines (Garamszegi et al. 2003), bill colour of zebra finches (Blount et al. 2003), complexity of the black bib of red-legged partridge (Pérez-Rodríguez et al. 2013) and the intensity of yellow plumage colouration of finches (Trigo and Mota 2015). The honesty of the signal is maintained as low-quality males are unable to afford the simultaneous costs of a strong immune response and expression of high quality SSC, thus leading to individual variation on sexual signalling (Fisher 1958; Grafen 1990). Most research focused on evaluating the sexual signalling quality and adaptive immunity, highlighting that SSC were indeed positively correlated with higher immune capacity. However, few studies tested these assumptions and trade-offs based on innate immunity, and no study has yet investigated the potential link between SCC and innate immunity in long distance migratory waders, which face both time and resource constraints throughout the annual cycle that are particularly acute during prenuptial migration and moult.

Black-tailed godwits (*Limosa limosa;* hereafter godwits) are long distant migratory waders that often fly long distances non-stop (+1000 km) to reach their breeding grounds (Alves et al. 2012; Alves and Lourenço 2014). This species has marked sexual dimorphism in terms of size and colouration (Gunnarsson et al. 2006; Schroeder et al. 2008; Gunnarsson et al. 2012), and their orange-brownish and black breeding plumage features are melanin-based (Toral et al. 2008). Furthermore, and as for all Charadriiformes, the expression of black coloured SSC is androgen-dependent (Bókony et al. 2008). Melanins are one of the most common animal colour pigments frequently implicated in social communication (Ducrest et al. 2008; Mcgraw 2008). As large endogenously synthetized proteins, these pigments occur in two main forms (Mcgraw 2005; Prota 1992): eumelanin provides the black, brown and grey colours; while pheomelanin gives origin to yellow and reddish-brown colours (Ducrest et al. 2008). The synthesis and final colouration of both colour pigments is controlled by the interplay of agonists and antagonist effectors of melanocortin receptors (MCR), especially MC1R (Mcgraw 2005; Ducrest et al. 2008). Besides involvement in social communication and sexual behaviour, there is currently strong evidence that melanocortin system exerts multiple immunomodulatory effects in animals and take part on anti-inflammatory, anti-oxidative and stress responses (Rózanowska et al. 1999; Mackintosh 2001; Catania 2007; reviewed in Ducrest et al. 2008). As reducers of oxidative and stress response and by favouring energy homeostasis, the physiological roles sustained by melanins can be adaptive for long-distance migrants during specific periods of their annual cycles. This is important because strenuous work generates oxidative stress (Von Schantz et al. 1999; Powers and Jackson 2010) and limited resources can energetically unbalance individuals (Alves et al. 2013). Migration can thus increase vulnerability to disease due to energetic and physiological demands (Sheldon and Verhulst 1996; Norris and Evans 2000; Hasselquist and Nilsson 2012; Pardal et al. *submitted* a), and also because individuals become exposed to a wider range of parasites (Møller and Erritzøe 1998; Møller et al. 2003; Altizer et al. 2011). Although melanins are generally implicated on immunosuppression of inflammatory responses (Catania 2007; Ducrest et al. 2008), their effects over the innate immunity on long-distance migrants remain unexplored. During prenuptial migration both sexes of godwits perform an extensive moult into colourful breeding plumage, in a period

when food resources are required to fuel their long flights and may not favour the investments of amino acids into plumage (Alves et al. 2013; Lourenço 2014).

By addressing these trade-offs in a species with considerable breeding plumage variation, our main goal was to assess SSC's signal honesty and how its variation relates to constitutive non-specific innate and adaptive immunity. To accomplish this, we first characterized the extension and colouration of breeding plumage by visual scoring of digital photos and quantified feather colouration through reflectance spectrophotometry. The levels of baseline innate and adaptive constitutive immunity were evaluated using three parameters: 1) circulating leukocyte (white blood cells) concentrations and relative proportions of cell types, providing general information on health status, stress and inflammatory processes (Davis et al. 2008; Clark et al. 2009); 2) activity levels of soluble plasma components, such as complement (measured has haemolysis) and natural antibodies (NAb´s) (measured has haemagglutination), which are responsible for facilitating phagocytosis of pathogens (Ochsenbein and Zinkernagel 2000; Matson et al. 2005); and 3) haptoglobin concentrations, which is an acute phase protein that limits microbial growth, and therefore a proxy for ongoing infection and inflammatory process (Matson et al. 2012a). Lastly, we explore how plumage SSC expression might influence these immune capacity metrics in a long distance migratory bird.

# Methods

*Study species*

Godwits have a widespread geographical range along the western Palearctic (Delany et al. 2009). They are ground-nesting birds, breeding in loose colonies in damp grasslands and both sexes incubate the egg clutch (Snow and Perrins 1998; Schroeder et al. 2009; Delany et al. 2009). Godwits present sex based size differences, and are also sexually dichromatic while in breeding plumage (Gunnarsson et al. 2006; Schroeder et al. 2008). Males are smaller, lighter and more colourful than females (Gunnarsson et al. 2006; Schroeder et al. 2008), and present a more extensive breeding plumage, with black belly stripes and a pallet of colours

that range from deep orange to rufous red on their neck and breast, and less white on the head than females (Gunnarsson et al. 2006; Schroeder et al. 2008). All three breeding plumage colours (black, orange and rufous red) are melanin-based (Mcgraw et al. 2005; Toral et al. 2008).

*Field sampling*

During the breeding season Icelandic godwits were captured in the Southern lowlands of Iceland (64°1'46.14"N, 20°59'6.04"W) in May-July 2013, while nominate godwits were captured in Friesland, northern part of The Netherlands (53°10'19.79"N, 5°46'35.60"W) in April-June 2014. To prevent nest abandonment capture was done using nest-traps 1-2 days' prior chick hatching, resulting in very short capture to release times and thus minimizing the impact on birds ($\bar{x}$ ±SD; 11.3 ±6.8 min, n=70). Birds were marked with a metal ring and a colour-ring combination, aged and measured. The following body size measurements were recorded: wing length (±1 mm), tarsus length (±0.1 mm), tarsus-toe (tarsus plus mid toe length without nail; ±1 mm), weight (±0.1 g), bill length (exposed culmen, ±0.1 mm), total head length (±0.1 mm), fat and muscle scores (Kaiser 1993; Bairlein 1994). Digital photos of ventral, dorsal and head profile were taken with a Canon Powershot SX50 HS camera using a white and grey background grid (Fig. 1) for assessment of breeding plumage features (see Table S1 for details). A sample of 5-6 coloured feathers from the same area of the chest (furcula region) were collected for spectrophotometric analysis of feather colouration. Feathers were kept in individual plastic bags, protected from humidity and abrasion and kept in the dark until analysis (see below). Digital pictures were randomized, scored blindly twice (by JAA) and finally assigned to individuals. In total we collected data from 70 breeding godwits.

**Figure 1. -** Example of digital photos used to score breeding plumage features of black-tailed godwits. From above to bottom and left to right: head profile to score bill colour extent and tone; dorsal position with and without open wing to count wintering feathers; ventral position to score baring and colour extent on the breast (see Table S1).

*Feather colour analysis*

To simulate the more natural colours and respecting their actual position on the bird breast, the 5 to 6 feathers previously collected were arranged in two superimposed rows on black cardboard cards, with two sets of 2 to 3 overlapping feathers being mounted for each individual prior to spectrophotometer measurements. Colour measurements were made with an Ocean Optics USB4000 Spectrophotometer (Ocean Optics, Dunedin, FL, USA), with a deuterium and halogen light source (Mikropack Mini-DT-2-GS, UV–VIS–NIR), emitting between 300 and 700 nm, attached to a Y fiber optic reflectance probe (Ocean Optics R400-7 UV–VIS) held vertically over the sample. The probe was attached to a rigid black support

to maintain distance between the probe and the sample (3 mm), and prevent entry of stray light. Three readings were taken per sampled area (most birds provided two samples N=61, and a few only one N=9), which were averaged for each bird. Readings of white (Ocean Optics, WS-1-SS White Standard) and black standards were made after every 5 individual sample measurements.

We used visual models to summarize the spectral data of each region into four quantum cone catches, corresponding to the four single cones found in the avian retina (Cuthill 2006). There is some variation between bird taxa on the peak of absorbance of colour cones with the majority of avian orders having the shortest wavelength cones in the near visible (VS) and a few others, including passerines, in the ultra-violet (UVS). The *Scolopacidae* are VS birds (Ödeen et al. 2010; Ödeen and Håstad 2013), thus we considered the typical near visible cone peaks: near visible (VS 402–426 nm), short (SWS 451–480 nm), medium (MWS 497–510 nm), long wavelength sensitive (LWS 543–571 nm), and the double-cone (DC) which is achromatic. Following Vorobyev et al. (1998), we calculated quantum catches for each of the four avian colour cones and the achromatic double-cone as the summed product of plumage reflectance, the ambient illuminance, and the absorbance spectrum of the cones across the wavelengths of the avian visual spectrum (300 to 700 nm; equation 1 in Vorobyev et al. 1998). In the model we included the cone quantum catches, standard daylight (D65) and ideal (white) background, and as reference, we used the peafowl cone sensitivity curves, representative of VS birds. The construction of visual models and gathering of colour variables were performed with PAVO software package, running on R (R Development Core Team 2013), developed by Maia et al. (2013).

*Blood sampling and baseline constitutive immunity assays*

For constitutive immunity assessment and molecular sexing, blood samples of ca. 320 µl were taken from the braquial vein of each individual, using non-heparinized microhematocrit capillary tubes. Two blood smears were immediately done, air-dried and stored for later processing, while blood for genomic DNA extraction was kept in eppendorf tubes with 96% ethanol, with the remainder of the sample being transferred to a clean eppendorf tube and kept refrigerated on ice until centrifugation. Centrifugation (6.000 rpm,

10 min) was done within the 2h-12h period following sampling with a portable field centrifuge in order to separate plasma from blood cells. Given that all samples were centrifuged within 12 hours, we have no reason to believe that post-sampling effects might have affected or final results (see Hoye 2012). Serum samples were then stored at -20ºC before transportation to the laboratory and then kept at -80ºC until analysis. For molecular sexing, total genomic DNA was extracted by standard ammonium acetate protocol, quantified in a NanoDrop (Thermo Scientific, Wilmington, USA) and diluted to working concentration (25 ng/μL). The sexing PCR amplified a fragment of CDH1 gene, using primers set 2550F (3´- GTTACTGATTCGTCTACGAGA-5´) and 2718R (3´-ATTGAAATGATCCAGTGCTTG-5´) following Fridolfsson and Ellegren (1999) protocol.

We quantified NAb´s and complement activity of lytic enzymes, following the haemolysis- haemagglutination assay described by Matson et al. (2005) with minor modifications. Briefly, serial dilutions of serum samples from each individual were run in duplicate (12.5 μl) on microtiter plates, along with chicken plasma standards (Probiologica, Lisboa, Portugal) and incubated in a 1% rabbit red blood cell (RBC) suspension (Probiologica, Lisboa, Portugal). Both lysis and agglutination scores reflect the last plasma dilution in the dilution series (*e.g.* rows 2 to 6) exhibiting each function. Scans of samples were randomized, scored blindly twice and later assigned to individuals (by SP). Haemolysis was identified by the presence of free haemoglobin and lack of intact cells, while haemagglutination was identified by clumped RBC. Both lysis and agglutination scores reflect the last plasma dilution in the dilution series (*e.g.* microtiter row 6) exhibiting either of these functions. To show partial haemagglutination or haemolysis, half scores were assigned. The within-plate variation and among-plates variation (mean ±SD), was 3.59 ±0.57 and 3.90 ±0.88 for haemolysis titres (respectively), and 9.37 ±0.63 and 9.26 ±0.39 for haemagglutination.

A commercial kit (Tridelta, Development Ltd., Maynooth, Ireland) was used to quantify the levels of circulating Hp-like proteins, following manufacturer´s instructions with minor modifications (extended standard curve to a more diluted range). Shortly, we mixed plasma and reagents in a 96-well microtiter plates and recorded absorbance at 620 nm after 5 min using a TRIAD plate reader (Triad Scientific, New Jersey, USA). Since

haemolysis of RBC can affect the result of the assay, visible hemolyzed samples or those that had previously given Hp values higher than (or equal to) 0.5 mg/ml (user defined threshold level) were run again and controlled for redness following the protocol described by Matson et al. (2012a). The among-plate variation was 0.34 ±0.01 mg/ml for control 1 and 1.26 ±0.38 mg/ml for control 2. For leukocyte counts, blood smears were stained by Hemacolor Rapid staining kit (Merck Millipore, Oeiras, Portugal), following manufacturer instructions. Microscope examination of blood smears was performed at 1000X magnification on an Olympus CX21 microscope (Olympus, Lisboa, Portugal) for leukocyte enumeration. Counts and leukocyte identification (lymphocytes, heterophils, basophils, monocytes and eosinophils) was made for the first 100 cells (Clark et al. 2009) by one observer (SP). The leukocyte density was determined by counting the numbers of WBC per 10.000 red blood cells (RBC) (equivalent to 25 vision fields at 1000X amplification) and the H/L ratio, which is the ratio between heterophils and lymphocytes (Davis et al. 2008), was also calculated after identification of 100 leukocytes.

*Data analysis*

Given the high level of sexual dimorphism in breeding plumage colouration of godwits, the relationships between immune parameters and plumage features were analysed separately for each sex. Body condition index was calculated as the standard residuals of the linear regression between body mass and tarsus length, therefore correcting for size variation among individuals. Some of the cellular immunity variables (WBC, H/L ratio and lymphocyte proportions) are known to suffer modifications after 30 min of handling due to capture stress (Buehler et al. 2008b), hence all individuals, except one male (blood sampled 55 min after capture and excluded from cellular parameters analysis), were sampled within 30 min ($\bar{x}$ ±SD; 10.5 ±4.1; range = 4.6-22.9). Consequently, the cellular immunity values are considered representative of the baseline values. As leukocyte variables were highly correlated (leukocyte proportions, WBC and H/L ratio), they entered a principal component analysis (PCA), using normally distributed data. Due to the fact that basophils were extremely rare and thus with no variation across individuals, these were excluded from the PCA. The analysis identified two PCs with eigenvalues >1 that cumulatively accounted for 66.0% of

male and 71.5% of female total variation of leukocyte variables (correlations of variables with PC´s axes for males and females are shown on Table 1). To capture the full extension and colouration of godwit breeding plumage, features such as bar and colour extension, colour and percentage of orange on the bill and number of wintering feathers on the back (Table S1), were integrated on a second PCA. The analysis identified two PCs for males and three for females, with eigenvalues >1 that cumulatively accounted for 60.5% of male and 76.7% of female total variation on plumage variables. Correlations of plumage variables with PC´s axes for males and females are presented on Table 1 and, as expected, the PC´s axes explained different features for each sex. Using both male and female data, the same procedure was executed for feather colouration parameters all of which had high factor loads on the PC. The PCA analysis for males and females altogether identified only one PC which accounted for 96.3% of the variance (Table S2). Individual scores for each principal component of all three PCA analyses, were used on subsequent analysis. For convenience, PCA cellular immunity axis were renamed has “Cell PC1” and “Cell PC2”, while for plumage PCA scores “SSC1” (PC1), “SSC2” (PC2) and “SSC3” (PC3) codes were used. Plasma baseline constitutive immunity variables were tested for collinearity using spearman rank correlation tests: for both males and females haemolysis was significantly correlated with haemagglutination; and for females, haemolysis was also correlated with cellular immunity Cell PC1 axis (data not shown). However, since correlations were all relatively weak ($r < 0.7$) all variables were kept for further analysis. None of the plasma variables and body condition were transformed as these were normally distributed.

**Table 1.** - Principal component analysis (PCA) loading factors and variance for the relationships between breeding plumage features and cellular immunity of male (n= 28) and female (n = 42) Black-tailed godwits, *Limosa limosa*. Bold values represent the variables that were significantly correlated with each axis.

| Variable | **Males** | | **Females** | | |
|---|---|---|---|---|---|
| *Plumage features* | *SSC1* | *SSC2* | *SSC1* | *SSC2* | *SSC3* |
| Bars extent | **-0.8788** | 0.2135 | **-0.8171** | 0.1704 | 0.0849 |
| Colour extent | -0.0821 | **-0.8530** | **-0.8189** | 0.1510 | -0.0756 |
| Bill (% of orange) | **0.5432** | -0.4062 | 0.0640 | **-0.8725** | -0.0486 |
| Bill colour | **-0.3696** | 0.2102 | -0.2813 | -0.2957 | **-0.8506** |
| Wintering feathers | **0.6460** | 0.6437 | 0.4259 | 0.5532 | -0.5368 |
| Variance (%) per component | 32.56 | 27.93 | 32.06 | 24.13 | 20.54 |
| Cumulative variance | 32.56 | 60.49 | 32.06 | 56.19 | 76.72 |
| *Cellular immunity* | *Cell PC1* | *Cell PC2* | *Cell PC1* | *Cell PC2* | - |
| Prop Lymphocytes | **-0.9318** | 0.2847 | 0.6654 | **-0.6964** | - |
| Prop Heterophils | **0.9317** | 0.1207 | **-0.9793** | -0.0658 | - |
| Prop Eosinophils | 0.1426 | **-0.8642** | 0.0771 | **0.8372** | - |
| Prop Monocytes | 0.1807 | **0.5373** | 0.1867 | **0.5102** | - |
| WBC | -0.0456 | **-0.3649** | 0.3384 | **-0.5744** | - |
| H/L | **0.9509** | 0.0185 | **-0.9662** | 0.1379 | - |
| Variance (%) per component | 45.17 | 20.83 | 46.81 | 24.69 | - |
| Cumulative variance | 45.17 | 66.00 | 46.81 | 71.50 | - |

We used generalized linear models (GLM) to test for the effects of constitutive immunity baseline and body condition (independent variables) on SSC (dependent variables) of males and females, with models run separately for each sex. We compared the fit and assessed residuals of models including all variables (full model), and using backward stepwise analysis to select only variables that presented the lowest Akaike Information Criterion (AIC). In all models, variables were treated has fixed factors. The majority of the fittest (best) models were those excluding cellular immunity variables, *i.e.* keeping only plasma variables (haemolysis, haemagglutination and haptoglobin) and body condition. To rule out the influence of body condition over SSC baseline immunity features, we also tested for the interaction between body condition and all immune variables for males and females. Statistical analyses were made in STATISTICA 10 (2011).

# Results

Both male and female godwits presented SSC features that appear to be mediated by constitutive baseline immunity parameters, specifically by the soluble components. For male godwits the colour extent was significantly affected by the level of circulating haptoglobin concentrations and haemagglutination activity (Fig. 2a & b). Considering that SSC2 axis indicates colour extent, our results show that males with a larger extent of orange (or reddish) colours, were also those that exhibited higher levels of haemagglutination and haptoglobin in the blood. In addition, this relationship was not mediated by male body condition as this had no significant effect on either immune parameter ( haemagglutination: $\chi2=2.810$, $p=0.094$; haptoglobin: $\chi2=1.424$, $p=0.233$). As a marginally non-significant result, breast feather colouration of males was negatively affected by body condition (Fig. 2c), as higher PCA values indicate less colour (supp. material Table S2). Hence males with chest feathers colour ranging from dark orange to reddish, were normally lighter. No other male SSC features were significantly influenced by plasma or cellular parameters (Table 2). For females, the extension of black bars and colour was significantly and negatively affected by haemolysis activity (Fig. 3), therefore, unlike males, females with larger extent of bars and coloured feathers exhibited lower levels of haemolysis. Again, this relation was not condition-dependent as the interaction between haemolysis and body condition was not significant (haemolysis*body condition, $\chi2=0.067$, $p=0.795$). No other female SSC features, was significantly influenced by baseline constitutive immunity parameters or affected by body condition (Table 2).

**Table 2. -** GLM results testing the effects of male and female black-tailed godwit breeding plumage features on constitutive baseline immunity. Significant results ($p<0.05$) are in bold and marginally non-significant results are underlined.

| **Males** | df | $\chi 2$ | *P* |
|---|---|---|---|
| *SSC1 (Bars extent, % of orange bill colour, bill colour & number of wintering feathers)* | | | |
| Body condition | 1 | 0.676 | 0.411 |
| *SSC2 (colour extent)* | | | |
| Haptoglobin (mg/ml) | 1 | 4.039 | **0.044** |
| haemagglutination titre | 1 | 5.849 | **0.016** |
| *Feather colouration* | | | |
| Body condition | 1 | 3.835 | <u>0.050</u> |
| **Females** | | | |
| *SSC1 (Bars and colour extent)* | | | |
| Haemolysis titre | 1 | 6.136 | **0.013** |
| haemagglutination titre | 1 | 3.477 | 0.062 |
| *SSC2 (% of orange bill colour & number of wintering feathers)* | | | |
| Body condition | 1 | 0.491 | 0.483 |
| *SSC3 (bill colour)* | | | |
| Body condition | 1 | 0.669 | 0.413 |
| *Feather colouration* | | | |
| Cell PC1 (Heterophils prop. & H/L index) | 1 | 1.735 | 0.188 |

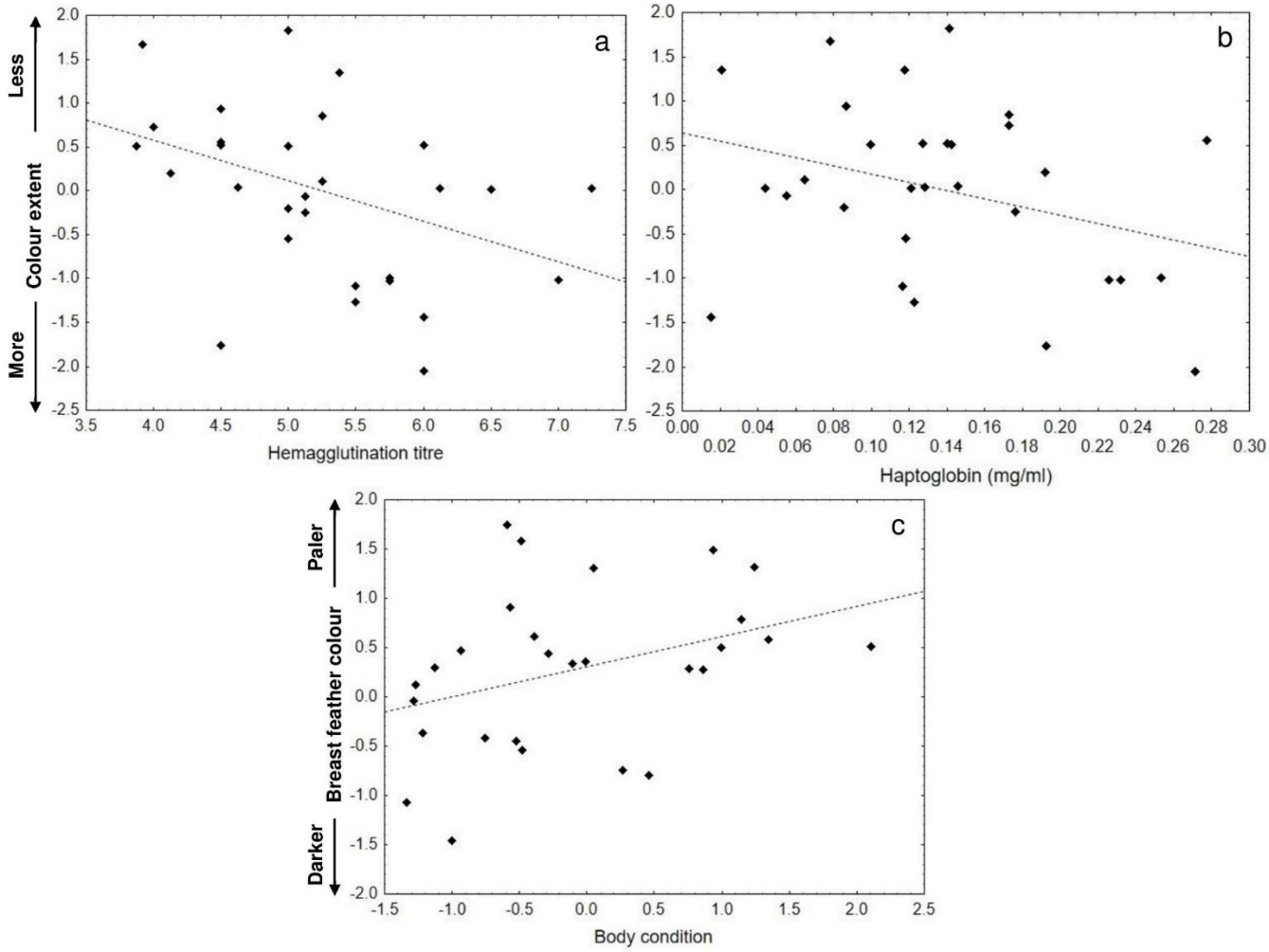



**Figure 2. -** Significant relationships between male black-tailed godwit breeding plumage features (SSC) and constitutive baseline immunity parameters: haptoglobin concentration a), haemagglutination titres b), and body condition c).

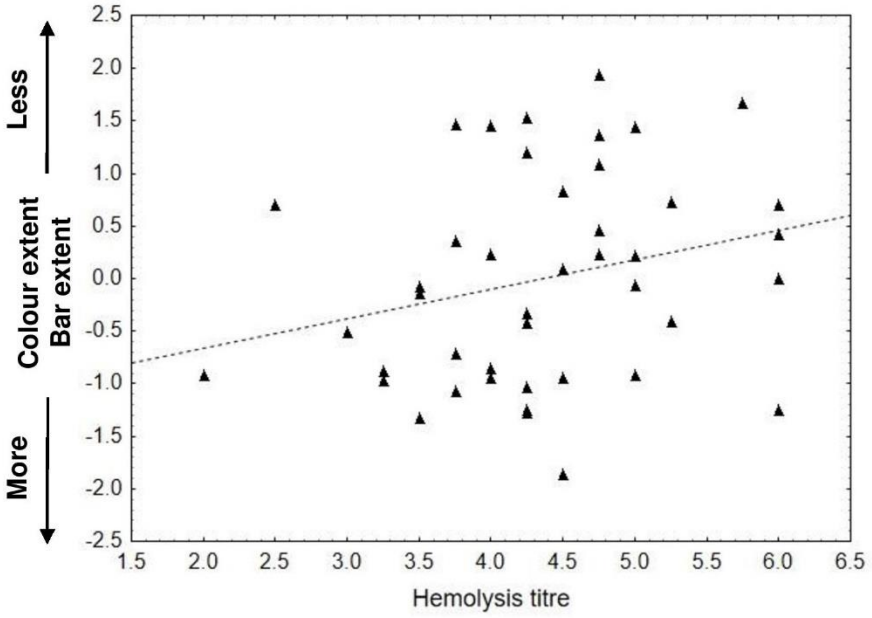



**Figure 3. -** Relationship between female black-tailed godwit breeding plumage features (SSC 1) and haemolysis titre.

# Discussion

By combining breeding plumage features considered SSC in godwits, and constitutive baseline immunity parameters, we showed that melanin-based features of male and female godwits are linked to soluble (but not cellular) parameters of innate and adaptive immunity and are also condition-dependent. The extent of colour (irrespective of tone) and black bars was negatively affected by the magnitude of innate immune responses, but the intensity of breast feather colouration was mediated by the body condition of males. Therefore, producing and maintaining SSC incurs costs in terms of body condition, and thus only males that are able to allocate the needed energy/resources are capable of maintaining both high-quality SSC and enhanced innate immunity. This supports the fact that SSC of male godwits are an honest signal of individual quality (Maynard Smith 1991; Zuk et al. 1996; Getty 2002). In males, the extension of the signal (*i.e.* colour extent) was associated with two immune components, the inflammatory mediators (haptoglobin, Fig. 2a) and constitutive adaptive immunity (*i.e.* NAb´s, Fig. 2b). However, this SSC signal was reversed in females, as

individuals with more extensive colour and bars, had lower complement activity (*i.e.* haemolysis, Fig. 3). Godwit breeding plumage features are largely the reflection of a higher proportion of phaeomelanin pigments (reddish-brown) and lower of eumelanin (brownish-black), and the differential production of both pigments are responsible for the existent plumage covariation between sexes. The produced amounts of each pigments are controlled by melanocortins (Ducrest et al. 2008), as the binding of melanocortin agonists to MC1R stimulates the production of black pigments, whereas binding of an antagonist, triggers the production of reddish-brown pigments. Since the melanocortin system has several pleiotropic effects due to binding of the same peptides to other four receptors with different functions and physiological effects (Ducrest et al. 2008), the final outcome over innate immunity and relation with SSC colouration and extension is difficult to predict. Based on the theoretical background of humans and other animals including birds, darker eumelanic individuals have better anti-inflammatory activity, lower cytokine levels and immunosuppressive effects over the proliferation of T and B-cells, than paler or phaeomelanic individuals (Cooper et al. 2005; Gonzalez-Rey et al. 2007; Ducrest et al. 2008). Our results are in accordance with literature, because the extension of the signal mediated by both pigments (black and orange) leads to a reduction on inflammatory mediators such as the complement in females (Gonzalez-Rey et al. 2007), while in males the signal mediated by one pigment (reddish) only, lead to immunoenhancement of pro-inflammatory haptoglobin proteins and NAb´s. As non-specific inflammatory mediators, haptoglobin concentrations and complement are those with the higher energetic and metabolic requirements (Lochmiller and Deerenberg 2000; Fox 2004; Rock and Kono 2008; Ashley et al. 2012), and thus under a tight regulation due to the risk of collateral tissue damage and oxidative stress. In godwits, males, but not females, seem to allocate energy and essential amino acids to both plumage and innate immunity in a period when energetic demands are high. These investments are not cost free, because mediation between immunity and sexual signalling is apparently nutritionally/condition dependent, as dark coloured males (dark orange or reddish) had significantly lower body condition than paler males, and again this relation was not recorded for females. Melanization degree over body condition effects were already reported on other wader species. For example, the enhanced black colouration of the Eurasian dotterel (*Charadrius morinellus*) and Ruff (*Philomachus pugnax*) (Höglund and

Lundberg 1989; Owens et al. 1994), translated into a higher body condition, while in their closest relative the Bar-tailed godwits (*Limosa lapponica*), also a long-distance migratory species, the rusty-red (most likely also melanin-based; Toral et al. 2008) breeding plumage colour and extension was shown to be condition-dependent, and an honest signal of individual quality (Piersma and Jukema 1993; Piersma et al. 2001). Thus, for both black- and bar-tailed godwits, only individuals which have sufficient energy/nutrients are able to afford investments into colourful plumage and innate immunity when still in migration, which potentially indicates their higher foraging skills, use of high-quality staging areas and/or better flying performances (Piersma and Jukema 1993).

Regarding the relationship between male colour extent and NAb´s, these molecules are an important link to the acquired immune defence, hence more specific and less costly to maintain (Buehler et al. 2008a). The prediction of the effects of melanocortins over humoral responses is not straightforward and no studies to date have shown a clear relationship between these peptides and antibody production. However, on a study by Gasparini et al. (2009) the degree of reddishness of female tawny owls (*Strix aluco*) was associated with humoral responses to Tetravac (against human pathogens) immunization, in which reddish females displayed higher levels of circulating antibodies than paler ones. Natural antibodies are a special kind of immunoglobulins with a broad specificity (Ochsenbein et al. 1999; Ochsenbein & Zinkernagel 2000). Despite their difference from induced antibodies such as those produced against Tetravac vaccine, their concentrations do not depend on pathogen stimuli and are constantly maintained. Nonetheless, both induced and constitutive antibodies are segregated by B-cells (Ochsenbein et al. 1999), therefore it seems likely that NAb´s are affected by melanization levels and melanocortin regulation in a similar way as specific antibodies.

The honesty of the SSC signal in godwits was not the same for the two sexes, because investment into plumage lead to sex-specific relationships between innate immunity parameters and SSC. Differences can be the consequence of differential predicted costs (development, maintenance and use) of each constitutive innate immunity parameters (Klasing 2004), driven by sex-biased energetic demands (Fitzpatrick et al. 1995), and/or energetically mediated by the migratory distances to breeding sites and limited time to

moult (Buehler and Piersma 2008). In long-distance migratory species that are energetically constrained, it seems likely that costly baseline immunity parameters are downregulated and traded-off against other physiological functions (Sheldon and Verhulst 1996), in this case breeding. Females bear the costs of egg production during times of nutritional stress that might occur upon arrival to breeding areas. This apparent relationship between plumage SSC and fecundity, may explain why females, in general, are not as brightly coloured as males (Fitzpatrick et al. 1995). It also points out that mate choice may not rely so much on the plumage features of females, but can be also dependent on other individual features such as size, as larger females lay larger eggs (Schroeder et al. 2009). When it comes to males, females are routinely viewed as the choosy sex, so males rely on their SSC to advertise their quality as potential mates. Despite being a monogamous species in which both male and female incubate the eggs and share parental care (Snow and Perrins 1998), male godwits still experience a strong intrasexual competition. When in search of females, more colourful and lighter wader males are preferred during aerial displays (Székely et al. 2000; Bókony et al. 2003; Székely et al. 2006), while when paired, godwit males defend their breeding territory prior to partner arrival (Gunnarsson et al. 2004).

In conclusion, the relationship between SSC plumage features and investment on constitutive innate immunity described in this study indicate that female godwits are not only selecting partners who proved to be more capable of combining moult and migration, but are also selecting males that are less vulnerable to novel infections. Because the immune tests chosen for this study are designed to measure "general immunocompetence", it remains to be tested wether the ability to fight-off novel pathogens in long-distance migratory species is also translated into a better ability to control re-infections by the same pathogens.

# Appendix

**Table S1. -** Plumage parameters of breeding black-tailed godwits accessed from ventral and dorsal photographs. Orange tones were given a standard Pantone code (PC).

| Plumage Score | Range | Description |
|---|---|---|
| **Bars extent** | 1-5 | 1= high chest to low chest (>25%), 2= low chest to upper belly (25-50%), 3= up belly to low belly (thy) (50-75%), 4= to legs (>75%), 5= black bars extended the legs and underneath the tail |
| **Color extent** | 1-5 | 1= neck (until the beginning of shoulders), 2= neck to high chest, 3= high chest to low chest, 4= low chest to up belly, 5= up belly to low belly |
| **Bill** | 0-100 | percentage of orange color along the bill |
| **Bill color** | 1-5 | 1= pink, 2= pink-orange, 3= pale orange, 4= orange, 5= dark orange |
| **Back wintering feathers** | Count | absolute number of wintering feathers on the back |

**Table S2. -** PCA results of feather colouration: v - near visible; s – short; m - medium and l - long wavelength sensitives; lum – luminosity.

| Variable | PC1 |
|---|---|
| *Feather colouration* | |
| v | **-0.9672** |
| s | **-0.9859** |
| m | **-0.9990** |
| l | **-0.9591** |
| lum | **-0.9944** |
| Cumulative variance | 96.28604 |

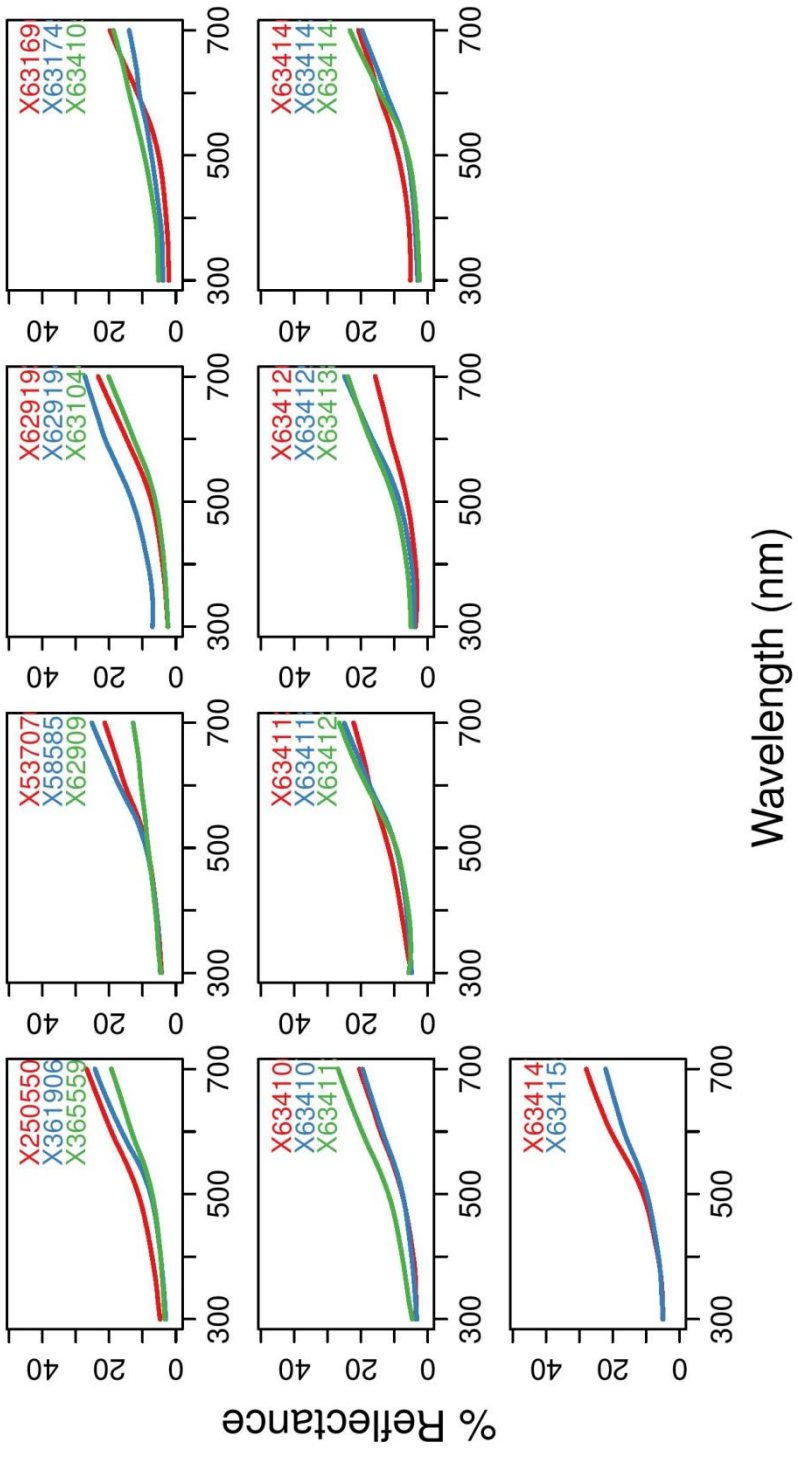



**Fig. S1. -** Reflectance spectra of melanin pigments. Each line corresponds to an actual reading on the feathers. Measurements were taken with Ocean Optics USB4000 spectrophotometer.

# Chapter V

## Indices of immune function used by ecologists are mostly unaffected by repeated freeze-thaw cycles and methodological deviations


**"The true method of knowledge is experiment."**

**William Blake**

# Abstract

1. Over the past couple of decades, measuring immunological parameters has become widespread in studies of ecology and evolution. A combination of different immunological indices is useful for quantifying different parts of the immune system and comprehensively assessing immune function. Running multiple immune assays usually requires samples to be repeatedly thawed and re-frozen. There is some evidence that repeated freezing and thawing can affect assay results, but this has never been comprehensively studied in some common ecological immunology assays.

2. We tested the effect of multiple (1, 2, 3, 4, 5, 10) freeze-thaw cycles on the results of four commonly used immunological assays: haemolysis-haemagglutination titres, haptoglobin concentration, bacterial killing capacity and total immunoglobulins (IgY). We tested five different bird species from four different bird orders (Passeriformes, Columbiformes, Charadriiformes and Galliformes), and we included both captive and free-living individuals. In addition, we tested for haptoglobin concentrations and the haemolysis-haemagglutination assay if re-analysing samples one year apart led to different results. For the haemolysis-haemagglutination assay we also tested two different sources of rabbit blood, and we compared untreated microtitre plates with plates that were "blocked" to prevent nonspecific interactions between the plate and assay reagents.

3. Repeated freezing and thawing of plasma had no effect on lysis titres, haptoglobin concentrations, bacterial killing capacity, or total immunoglobulin levels. Agglutination titres were unaffected for up to five cycles but were lower after ten freeze-thaw cycles. For the haemolysis-haemagglutination assay and haptoglobin concentrations, re-analysing samples one year apart yielded highly correlated data. For the haemolysis-haemagglutination assay, the source of rabbit blood did not influence the results, and the untreated *vs.* blocked plates differed slightly overall, but at the individual level assay results were highly correlated. Using different rabbit blood sources or different types of microtitre plates yielded highly correlated data.

4. Our data suggest that repeated freeze-thaw cycles do not impair assay results to the point of influencing ecological or evolutionary conclusions. Plasma samples can be safely stored in one tube and thawed repeatedly for different assays. Nevertheless, we recommend consistent treatment of samples in terms of freeze-thaw cycles or other laboratory treatments to minimize the potential for introducing a systematic bias.

**Keywords:** eco-immunology, immunity assays, assay methodology, avian, pre-analytical error, sample stability, repeated defrosting

# Introduction

In the mid 1990´s, evolutionary ecologists began to consider that the costs and benefits associated with the immune system might lead to important trade-offs between defences against diseases and other behavioural and physiological processes that impact individual fitness (Sheldon and Verhulst 1996; Råberg et al. 1998; Norris and Evans 2000). Since then, several techniques to quantify immune function have become available, and measuring different immunological parameters in free-living and captive animals has become widespread in studies of ecology and evolution (Figure 1). In fact, given the complexity of the immune system, a combination of assays targeting different parts of the immune system is favoured in order to gain an understanding that is as comprehensive as possible. Numerous publications have stressed the importance of measuring more than one immune parameter (Adamo 2004; Martin et al. 2006; Salvante 2006; Matson et al. 2006b; Boughton et al. 2011; Demas et al. 2011; Pedersen and Babayan 2011).

Although multiple immune assays are potentially available when testing immune function (Adamo 2004; Salvante 2006; Millet et al. 2007), a few assays to measure baseline immune function are particular widespread in ecological and evolutionary research. The haemolysis-haemagglutination assay (HLHA) allows the quantification of complement (measured as lysis titres) and natural antibodies (measured as agglutination titres) (Matson et al. 2005). Other assays measure an acute phase protein (haptoglobin) concentrations (Matson et al. 2006b; Hegemann et al. 2012a; Matson et al. 2012a), bacterial killing capacity (Matson et al. 2006a; Liebl and Martin 2009; French and Neuman-Lee 2012) and total immunoglobulin (IgY) levels (Grindstaff et al. 2006). All of these immunological assays can be done using previously frozen plasma (or serum) samples. This advantage, combined with the assays relative simplicity and inter-specific utility, has translated to a widespread application in ecological and evolutionary studies (Figure 1); especially in studies where logistical constraints (often related to working with small free-living animals) pose challenges. In fact, many studies try to combine at least two or more of those assays to get a more comprehensive view on immune function (Palacios et al. 2011; Nebel et al. 2013; Versteegh et al. 2014; Horrocks et al. 2015; van Dijk et al. 2015; Hegemann et al. 2015).

Running multiple immune assays on the same individual often results in samples that experience multiple freeze-thaw cycles. Freeze-thaw cycles can degrade samples by modulating the chemical composition and molecular function (Lee et al. 2015). For example, in human and other mammals, some common clinical chemistry analytes are affected by repeated defrosting, while others remain stable even after multiple freeze-thaw cycles (Reynolds et al. 2006; Kale et al. 2012; Cuhadar et al. 2013). This opens the questions whether repeated defrosting also impacts assay results used for studies in ecology and evolution. Yet, to the best of our knowledge, data on whether freeze-thaw cycles impair the results of many commonly used assays in ecological immunology are not available. One exception is the assay of bacterial killing capacity in which data suggest that repeated freezing and thawing lead to decreased killing (Liebl and Martin 2009).

Here, we present a comprehensive study testing if repeated freeze-thaw cycles influence the results of four immune assays (haemolysis-haemagglution titres, haptoglobin concentrations, bacteria killing capacity and total immunoglobulin levels) frequently used in studies of ecology and evolution. We used samples from five bird species representing four different orders. Furthermore, we also systematically examined several other methodological concerns. With haemolysis-haemagglutination titres and haptoglobin concentrations, we also explored the relationships between results from duplicate intra-sample analyses that were separated by a year. Lastly, we tested if lysis and agglutination titres depended on the source of rabbit red blood cells (used as an antigen-rich particulate) or on microtitre plate characteristics.

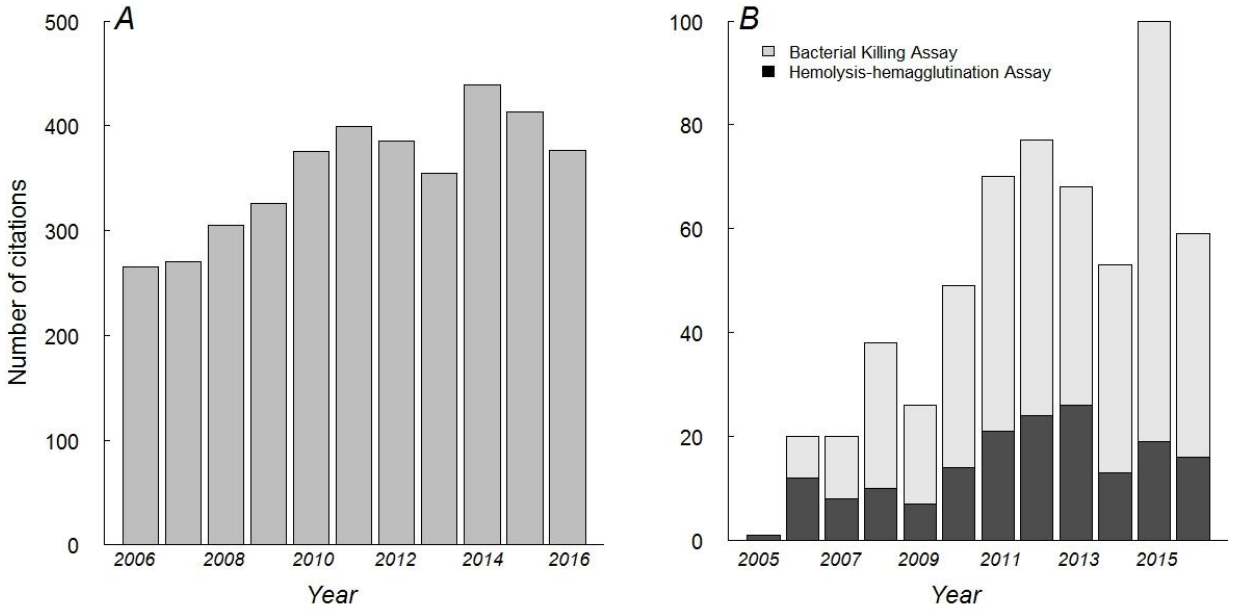



**Figure 1. -** Ecological immunology techniques have become important tools to test hypotheses in ecology and evolution over the past decade. A) Annual number of citations from 2006 to 2016 for 10 influential ecological immunology papers published between 1996 and 2006 (Sheldon and Verhulst 1996; Råberg et al. 1998; Norris and Evans 2000; Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; Schmid-Hempel and Ebert 2003; Schmid-Hempel 2003; Klasing 2004; Lee 2006; Altizer et al. 2006). B) Annual number of citations for the methods papers introducing two commonly used methods in eco-immunology: the haemolysis-haemagglutination assay (Matson et al. 2005) and the bacteria killing assay (Tieleman et al. 2005; Matson et al. 2006a; Millet et al. 2007; Liebl and Martin 2009; French and Neuman-Lee 2012). All number of citations are based on a web of science search completed on 24 January 2017.

# Methods

*Sample collection and storage*

We sampled five different bird species from four different orders: free-living adult and nestling Jackdaws (*Corvus monedula*) from a study colony in Sweden, adult free-living Common Blackbirds (*Turdus merula*) and Ruffs (*Philomachus pugnax*) from Portugal, adult Homing pigeons (*Columba livia f. domestica*) from a captive population in the Netherlands (Matson et al. 2012a), and Lohman White breed chickens (*Gallus gallus domesticus*) from a

population housed at Lund University (Olsson et al. 2016). We collected blood samples from the brachial vein using heparinised microhaematocrit capillary tubes or syringes (in case of pigeons). Samples were chilled until centrifugation to separate the cellular and plasma fractions. After centrifugation several individual samples were pooled (within species and age class) and then divided into a maximum of 6 different aliquots and frozen. We made several series of up to 6 different aliquots for the four different immune assays that we tested. For Blackbirds and Ruffs, samples were divided into different aliquots after an initial freeze; hence, the first freeze-thaw cycle (see below) is missing for these species.

*Experimental set-up*

Samples were moved from a -20ºC freezer to a lab bench to thaw at room temperature for 30 minutes. All samples were completely melted after this period. Samples were then re-frozen at -20ºC degrees for at least 6 hrs before the next freeze-thaw cycle began. We produced aliquots with 1, 2, 3, 4, 5 or 10 freeze-thaw cycles for each of the four immune assays. The samples with only one freeze-thaw cycle were collected, frozen, thawed, and then directly analysed together with all other samples, *i.e.* the samples undergoing 2, 3, 4, 5 or 10 freeze-thaw cycles. Hence, storage age is equal for all freeze-thaw cycles within a species. Prior to analyses, all samples were randomized. Samples were run as triplicates (Jackdaws, Chicken, Ruffs, Blackbirds) or duplicates (Pigeons) within the same assay microtitre plate.

*Haptoglobin*

Haptoglobin is an acute phase protein that is released from the liver during a pathogenic challenge (Quaye 2008). In Jackdaw, Pigeon, Chicken and Blackbird samples, we quantified concentrations (mg mL−1) of this protein (or a functional equivalent) in plasma samples using a commercially available colorimetric assay kit (TP801; Tri-Delta Diagnostics, NJ, USA). This functional assay quantifies the heme-binding capacity of plasma. We followed the 'manual method' instructions provided by the kit manufacturer with minor modifications following Matson et al. ( 2012a). We measured absorbance at three wavelengths (405, 450 and 630 nm) prior to the addition of the final reagent that initiated the colour-change

reaction. We measured absorbance (630 nm) prior to the addition of the final reagent that initiated the colour-change reaction. We used the pre-scan at the normal assay wavelength of 630 nm to correct for differences in plasma colour and cloudiness by subtracting pre-scan absorbance values from final absorbance values. We used the 405 and 450nm pre-scan to statistically analyze and correct for differences in plasma sample redness, an indication of haemolysis, which can affect the assay (Matson et al. 2012a). As our different aliquots were made of the same pool and hence consisted of similar plasma redness, neither of those two wavelengths (405 and 450nm) explained any variation and we do not report on those measurements any further.

*Bacteria killing capacity*

In Jackdaw, Blackbird and Ruff samples, we quantified the capacity of plasma to kill *E. coli* using the method described by French and Neuman-Lee (2012) with a few modifications. We mixed 3 µl of plasma with 4 µl of $10^5$ *E. coli* solution for Blackbirds and Ruffs, and we mixed 2 µl of plasma with 4 µl of $10^6$ *E. coli* solution for Jackdaws. Microtitre plates were incubated at 37°C for 12 hrs. We measured bacteria growth at 600 nm using a microplate reader (Eikenaar and Hegemann 2016). To calculate bacteria killing ability, we first subtracted background absorbance readings from the 12 hrs absorbance readings. We then calculated the percentage of *E. coli* killed relative to the growth of *E. coli* in the positive controls as follows: one minus the mean absorbance for each sample, divided by the mean absorbance for the positive controls (wells containing only bacteria and broth, run in quadruple per microtitre plate), multiplied by 100. We used four negative controls per plate to ensure that there was no contamination.

*Haemolysis-haemagglutination*

Using Jackdaw, Ruff, pigeon and chicken samples, we quantified complement (measured as lysis titres) and natural antibodies (measured as agglutination titres) following the method of Matson et al. (2005). In brief, plasma samples were serially diluted in microtitre plates and incubated with a 1% rabbit red blood cell suspension (Harlan Laboratories, United

Kingdom). Following incubation, plate images were recorded after 20 min (agglutination, all species), 90 min (lysis, all species except pigeon), and 24 hrs (lysis, pigeon). Images of individual samples were randomized and scored at least two time, always blindly with respect to sample identity and freeze-thaw cycle number. Lysis and agglutination were recorded as titres ($-\log^2$ of the last plasma dilution that shows each reaction).

*Total immunoglobulins*

We quantified the total level of antibodies (immunoglobulins IgY; the bird equivalent to IgG in mammals), in plasma by means of an enzyme-linked immunosorbent assay (ELISA) following the protocol described by Sköld-Chiriac et al. (2014). Since this protocol is for passerines, we analysed only Jackdaw and Blackbird samples. In brief, microtitre plates were coated with goat-anti-bird immunoglobulin and blocked with 3% powdered milk PBS/Tween 20. We added 100 µl of plasma diluted 1:300 in 1% powdered milk in PBS/Tween 20. We added 100 µl of rabbit-anti–Red-winged Blackbird IgG (1:1000 dilution in 1% powdered milk in PBS/Tween 20). Afterwards we added 100 µl of peroxidase labelled goat-anti-rabbit antibody (1:2000 dilution in 1% powdered milk in PBS/Tween 20). Lastly, we added 100 µl of ABTS (Sigma-Aldrich, catalogue no. A1888) and peroxidase diluted in citrate buffer. Antibody concentrations are represented as the slopes of colour change of the substrate overtime (measured in $10^{-3}$ optical density per minute [mOD $min^{-1}$]). Ultimately, antibody levels are calculated as the mean of the duplicates minus the mean of the blanks and are transformed based on the plate standards to correct for variation among plates.

*Other methodological considerations*

In addition to analysing the series of freeze-thaw cycles under standard assay conditions, we conducted additional tests concerning haptoglobin concentrations and lysis and agglutination titres. First, we quantified haptoglobin concentrations and lysis and agglutination titres in 32 pigeon samples that were collected over the course of 2013. Using the standard procedures, we analysed these samples first in the summer of 2014 and analysed them again in the summer of 2015; samples were stored at -20 degree Celsius.

Second, we concurrently quantified only agglutination in 61 samples from pigeons (housed at University of Kiel, Germany; Matson et al. 2012b) using the standard protocol but with rabbit red blood cells from two different suppliers (Harlan, as above, and Hemostat Laboratories, CA, USA). Third, we also analysed 72 pigeon samples using the standard haemolysis and haemagglutination protocol but with untreated microtitre plates (*i.e.* standard protocol) and plates that were blocked to prevent nonspecific interactions between the plate and assay reagents. After the blocking procedure, which used a 2% powdered milk solution in PBS for 1 hour at room temperature, plates were washed three times with PBS/Tween 20 (Vermeulen et al. 2015).

*Statistical analyses*

To test for the effects of freeze-thaw cycles on the immune parameters, we used linear mixed models (function lme of package nlme; Pinheiro 2016) in R version 3.2.3. Immune parameters served as dependent variables. We included species, freeze-thaw cycle and the interaction between the two as explanatory variables, and sample identity as a random effect. Assumptions of all models were checked on the residuals of the final model.

For the other methodological analyses, we used paired comparisons to test group differences and correlations to evaluate patterns among individuals. Parametric and non-parametric tests gave qualitatively similar results; here we report the parametric test statistics.

# Results

*Repeated freeze-thawing cycles*

Repeated freezing and thawing of plasma had no effect on haptoglobin concentrations, lysis titres, bacteria killing capacities or immunoglobulin levels (Table 1; Figure 2). Agglutination titres declined with increasing freeze-thawing cycles, but this effect was only seen after ten freeze-thaw cycles. When the ten cycles point was excluded, the effect of freeze-thaw cycle on agglutination titres disappeared ($F = 1.29$, $df = 1,45$, $p = 0.262$). The interaction between species and freeze-thawing cycle was never significant (Table 1).

**Table 1. -** Statistics and coefficients of the linear mixed models of different immune parameters in relation to repeated freeze-thawing cycles. P-values < 0.05 are in bold.

| ***Freeze-thaw cycle*** | **Haptoglobin conc.** | **Lysis titre** | **Agglutination titre** | **Bacteria killing capacity** | **Total IgY level** |
|---|---|---|---|---|---|
| F | 1.44 | 2.94 | 17.88 | 0.27 | 0.04 |
| β | 0 | -0.01 | -0.1 | -0.146 | 0.04 |
| df | 1,57 | 1,56 | 1,56 | 1,17 | 1,13 |
| p | 0.235 | 0.092 | **0.001** | 0.61 | 0.842 |
| ***Species*** | | | | | |
| F | 9.89 | 80.69 | 5.56 | 9.95 | 1.49 |
| df | 3,8 | 3,8 | 3,8 | 2,1 | 1,1 |
| p | **0.005** | **<0.001** | **0.023** | 0.219 | 0.437 |
| ***Freeze-thaw cycle * species*** | | | | | |
| F | 4.53 | 5.73 | 7.05 | 1.21 | 1.29 |
| df | 3 | 3 | 3 | 2 | 1 |
| p | 0.21 | 0.126 | 0.07 | 0.546 | 0.256 |

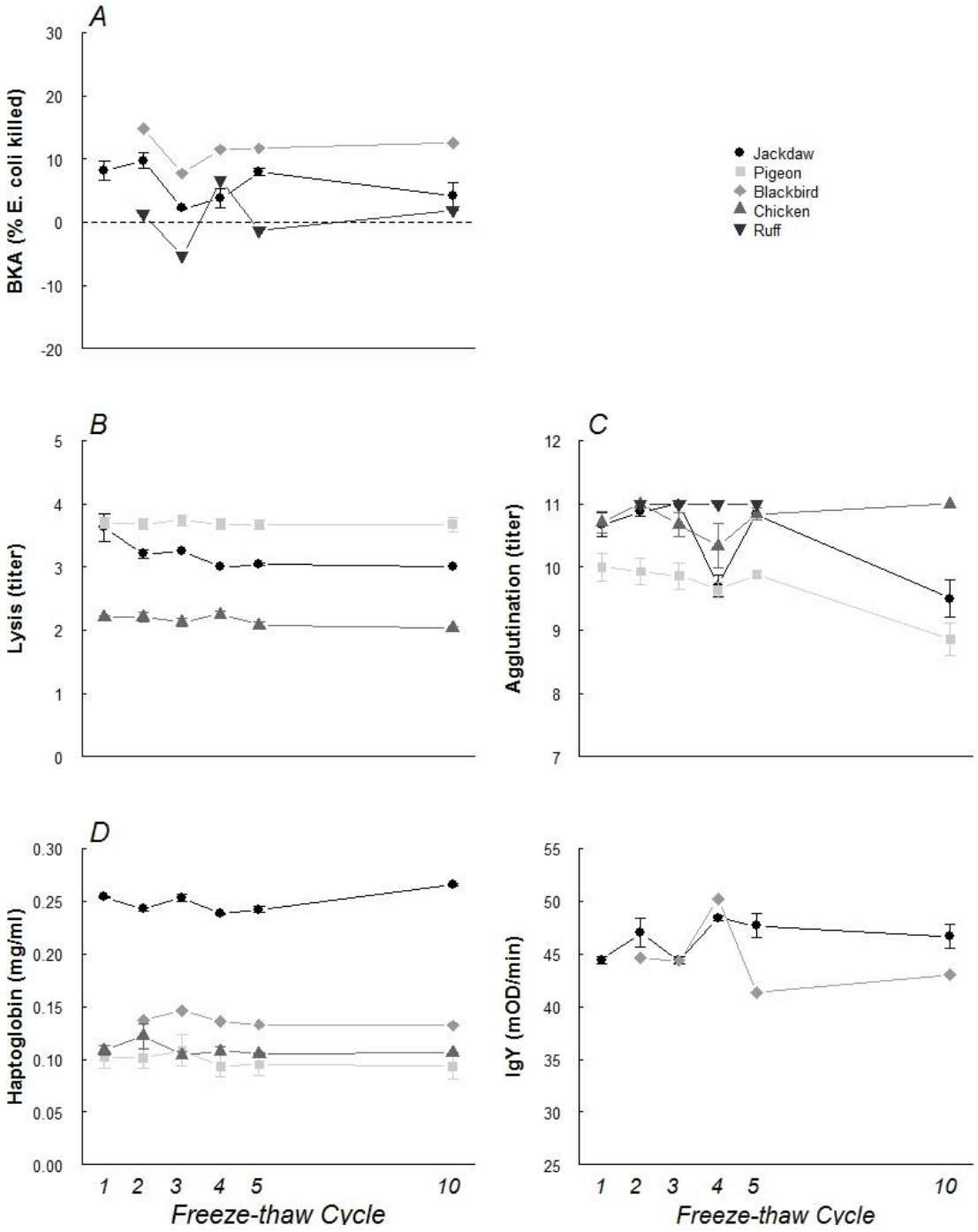



**Figure 2. -** Effects of repeated freeze-thaw cycles on five commonly applied immune parameters in ecology and evolution. A) bacteria killing capacity, B) lysis titres, C) agglutination titres, D) haptoglobin concentrations and E) Immunoglobulin levels. Symbols depict means and standard errors.

*Other methodological considerations*

Re-analysis of samples after one year of storage resulted in significantly lower haptoglobin concentrations (mean Hp in 2014 = 0.127; mean Hp in 2015 = 0.117; t = 2.24, df = 31, $p$ = 0.033). Across samples, haptoglobin concentrations were correlated between the two years (R=0.93, t = 14.07, df = 30, $p < 0.001$). Re-analysis of samples after one year of storage resulted in significantly lower lysis titres (mean lysis for 2014 = 1.8; mean lysis for 2015 = 1.0; t = 4.71, df = 31, $p < 0.001$). Across samples, lysis titres were correlated between the two years (R=0.78, t = 6.89, df = 30, $p < 0.001$). Finally, re-analysis of samples after one year of storage lead to similar agglutination titres (mean agglutination for 2014 = 7.5; mean agglutination for 2015 = 7.4; t = 0.13, df = 31, $p$ = 0.90). Across samples, agglutination titres were not correlated between the two years (R=0.15, t = 0.80, df = 30, $p > 0.4$).

The comparison of the two different sources of rabbit blood revealed no effect on agglutination titres (mean agglutination for Harlan = 3.8; mean agglutination for Hemostat = 3.9; t = 1.25, df = 60, $p > 0.2$). Across samples, agglutination titres using the two sources were highly correlated (R=0.68, t = 7.07, df = 59, $p < 0.001$).

Blocked microtitre plates did not differ significantly from unblocked plates in terms of lysis titres (mean lysis for blocked = 3.5; mean lysis for unblocked = 3.4; t = -0.51, df = 71, $p > 0.6$). Across samples, lysis titres arising under the two plate conditions were highly correlated (R=0.86, t = 14.12, df = 70, $p < 0.001$). Blocked plates resulted in significantly higher agglutination titres compared to unblocked plates (mean agglutination for blocked = 8.0; mean agglutination for unblocked = 7.5; t = -2.51, df = 71, $p$ = 0.014). Across samples, agglutination titres arising under the two plate conditions were highly correlated (R=0.60, t = 6.24, df = 70, $p < 0.001$).

# Discussion

Measuring immune function in wild animals is constrained by many logistical challenges (Boughton et al. 2011; Pedersen and Babayan 2011). Over the last two decades, however, several new assays have become available (Matson et al. 2005; Millet et al. 2007; French and Neuman-Lee 2012). Since these assays rely on blood plasma (or serum), which is often collected in small volumes and frozen in microcentrifuge tubes, post-sample-collection handling might introduce variation into assay results. We found clear evidence that one such handling concern--repeated freezing and thawing--had virtually no effect on the final outcome of four assays currently and widely used to study ecological immunology. Only agglutination decreased at the point of ten freeze-thaw cycles. This near absence of effects of repeated freeze-thaw cycles on the results of the immune assays is reassuring for investigators who wish to measure several indices of immune function in single samples from individual animals. Since the stability of samples was consistent across species from four different taxonomic orders and in free-living and captive birds, the resistance to freeze-thaw effect appears to be independent of the species and condition of the sampled individuals. Although we have only tested birds, we can offer no clear reason why results would differ for other vertebrate classes (*e.g.* Mammalia, Reptilia, etc.), but this remains to be tested. For human samples, repeated freezing to -70 °C and thawing has no meaningful effects on the plasma and serum concentrations of a considerable number of micronutrients and hormones (Comstock et al. 2001).

Our current results are in line with other studies that found no or little effect of multiple freeze-thaw cycles on different chemical analytes of blood (Comstock et al. 2001; Reynolds et al. 2006; Kale et al. 2012; Cuhadar et al. 2013). However, some studies have suggested freeze-thaw effects, including on parameters we measured here. For example, Boadella and Gortázar ( 2011) advise against carrying out ELISAs with samples with medium to strong haemolysis that have undergone more than three freeze-thaw cycles and with any samples that have undergone more than five cycles. Yet our study and the study of Pinsky et al. ( 2003) failed to find any effect on the antibody levels of up to ten freeze-thaw cycles. Gutiérrez et al. (2011a) found that haptoglobin concentrations increased significantly by

29% after five freeze-thaw cycles, but in that study they used a time-resolved immunofluorometric method, in contrast to our functional colorimetric assay which is commonly used in ecological studies. Thus, the different sensitivity to freeze-thaw might result from different methods. Furthermore, different freezer storage temperatures might play an important role on sample degradation and help to explain some of the variation in results among studies. In general, the colder the storage temperature, the smaller the degradation effect (Sgoutas and Tuten 1992).

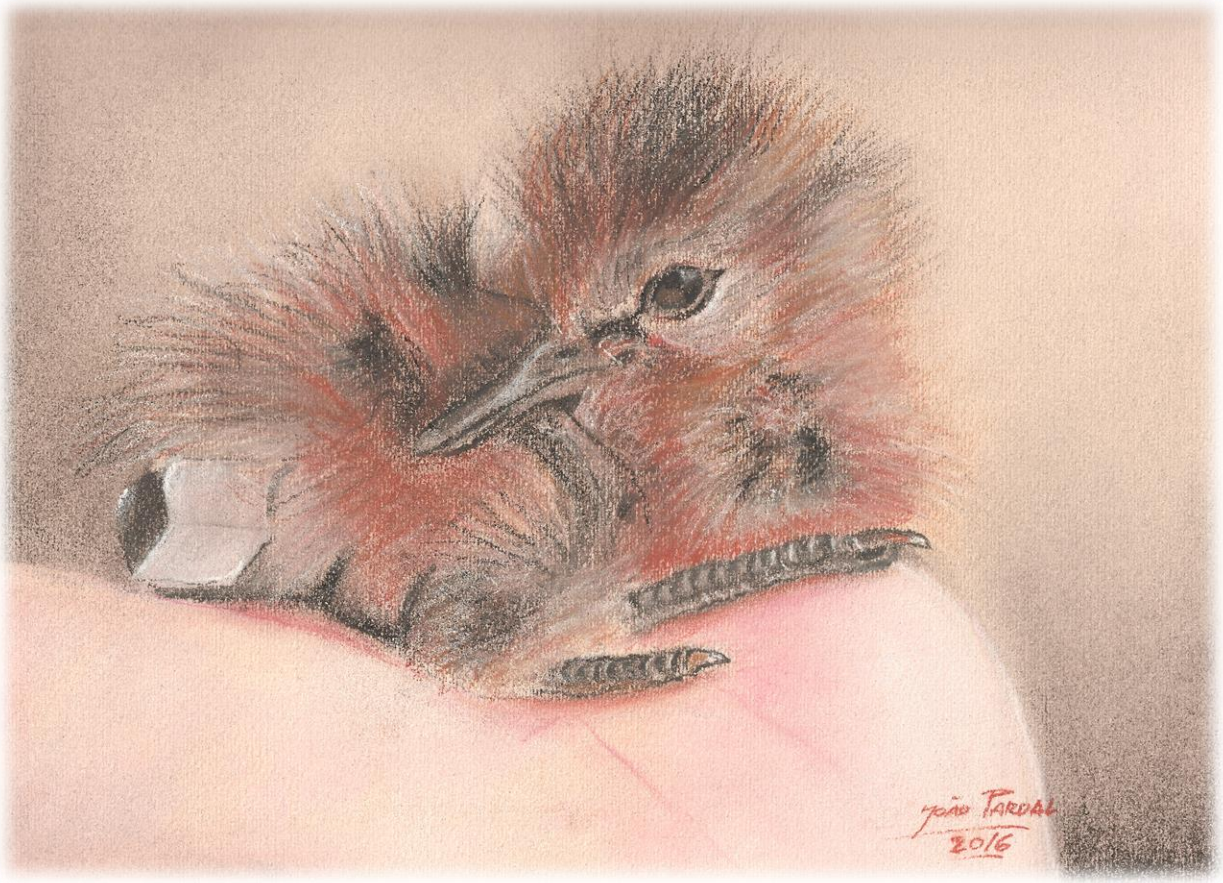
Because storage time in a freezer might also impact the results of immunological assays, we investigated this possibility with haptoglobin concentrations and for lysis and agglutination titres, albeit in a less detailed manner than with our freeze-thaw analyses. Re-analysis of samples after one year of storage at -20°C, generally resulted in values that were significantly correlated with values from the previous year. Such correlations were found for haptoglobin and lysis, but with both of these indices, one year of storage resulted in overall declines. In the case of haptoglobin, the between-year differences were not simply a by-product of between-year differences in the standard curves used to calculate concentrations; raw absorbance values (*e.g.* "blueness" measured at 630nm 5 minutes after colour change initiation) also showed between-year differences and correlations across samples. The correlations we observed suggest that biological patterns at the individual level should be discernible even if samples have aged somewhat. Inclusion of sample age or a factor related to analysis batch in statistical analyses could help account for the decline due to storage time. However, lab analyses of samples that differ both in age and another factor (*e.g.* source population identity) that is both of study interest *and* that is confounded with sample age should probably be avoided. In contrast to the other indices, agglutination showed no overall decline due to storage, but across samples, values were uncorrelated between the two years. The well-known relative durability of antibodies (compared to, *e.g.* lytic enzymes) probably underlies the resistance of agglutination to freezing and thawing (Fipps et al. 1988; Argentieri et al. 2013). The mechanisms underlying agglutination are even resistant to heat treatment designed to inactivate complement in plasma samples (Matson et al. 2005). The absence of a correlation between agglutination titres scored from first and second analyses raises questions about the value of this index in studies aimed at explaining variation among

individuals. The initial description of this assay focused on differences among groups (species, Matson et al. 2005), and further study of its use at the individual level is needed.

Tests aimed at the origin of the rabbit red blood cells and at a characteristic of the assay plates, two other potential methodological sources of variation in the haemolysis-haemagglutination assay, also provided new insights into this assay. The two types of rabbit blood did not differ in agglutination titres (lysis was absent in these assay bouts) and sample values were highly correlated, suggesting that researchers working in different regions of the world with different accessibility to and markets for biological products should be able to compare assay results, if all else is equal. Assay plates might also differ in terms of non-specific binding depending on manufacturer (Anne Peters, *pers. comm*.). To systematically gain insight into possible effects of non-specific interactions between antibodies, the red blood cell targets, and the plastic plates, we compared normal plastic assay plates to ones that had been blocked with powdered milk. As would be expected, blocking had no impact on lysis; however, blocked plates had higher agglutination titres on average. Effects of blocking are an unlikely source of variation within an individual study or lab (*i.e.* all samples should be analysed using the same source of plates, which have been similarly handled). But blocking and potentially other plate characteristics could influence the results of agglutination and complicate comparisons among studies, particularly if absolute levels (and not intra-study patterns) are being compared.

To conclude, our data suggest that several commonly used ecoimmunological assays produce consistent results even in the face of several factors that could potentially introduce variation or otherwise influence results. Most notably, repeated freeze-thaw cycles do not impair assay results or study conclusions. Thus, plasma (or serum) samples can be safely stored in one tube and thawed (at least 5-10 times) as needed for different assays. Still, best laboratory practices dictate standardizing the freeze-thaw cycles, the assay order, and other assay parameters to the greatest extent possible.

# General Discussion




**“The learned man knows**

**that he is ignorant.”**

**Victor Hugo**

In this thesis, we studied the consequences of the ecology and life-history strategies of Black-tailed godwits (*Limosa limosa*) on the physiology of immune response. During their annual-cycle Icelandic godwits have a lower risk of contracting infections, while nominate godwits have a higher risk. Specifically, we focused on how differential pathogen-pressure, physiological trade-offs and sexual selection mediate the innate and adaptive immune response. Moreover, we provide new insights about the variation of immune function along the flyway of long-distance free-living migratory birds. To address these points we took an integrative research approach, making use of tools and methods that are normally used and developed for other research areas and contexts. The final results obtained in this thesis are a valuable contribution to understanding the mechanisms mediating host and pathogen interactions and later outcomes in terms of natural selection.

I will start by summarizing my main results and bring these findings into a broader context of immune responses, physiological trade-offs and context of what is known for this study model. I will also focus attention on the ecological changes that are driving the decline of many Afro-Palearctic migrant bird populations and suggest future research avenues and open questions regarding immune function.

## Chapter I: The innate immune defence annual rhythm and response to the environment

In chapter I, I wanted to test Piersma hypothesis about the differential abilities of waders to fight disease, that may vary according to their migratory strategies (Piersma 1997, 2003). I showed that innate baseline immunity is not a static, but rather a dynamic physiological component during the annual cycle of the godwits, that changes according to season and available resources, due to physiological and environmental constraints. These immunologic adjustments appear to be subspecies specific, because the trends I see do not only reflect the unique physiological and energetic trade-offs, but they also vary according to the disease landscapes that individuals from the different subspecies experience.

The results obtained corroborate other studies showing an overall immunosuppression of the immune system during migration regardless of the species or subspecies (*e.g.* Buehler et al. 2010; Hegemann et al. 2012a; Versteegh et al. 2014; Eikenaar and Hegemann 2016), and this suppression leads to a period of higher vulnerability to disease. Migration exerts strong energetic and physiological demands and interestingly, the effects of suppression of the immune responses were stronger during the autumn than spring migration. In nominate godwit species, these differences in overall immunosuppression between migration periods could be explained by the overlap of two costly physiological processes (migration and extensive moult), alongside with immunosuppressive remnants of breeding hormones or even a possible carryover effect of the parental effort during the breeding season (Hegemann et al. 2013a). The immunosuppressive effects of migration also indicate that individuals in the nominate subspecies did not enhance the immune system and/or are energetically/physiologically incapable of doing so, as an anticipation for the higher parasitic pressure experienced on tropical areas of Guinea-Bissau.

The environmental differences that each subspecies experiences in pathogen pressure, rather than physiological or genetic components, seem to determine the immune strategy used and differences seen at the investment level on innate immunity, especially those related to inflammation. The trade-offs between immune function and competing physiological components become more apparent during energetically demanding periods, which in this case was the breeding season rather than the wintering period. During breeding, Icelandic godwits downregulated costly components of the immune function, thus supporting the hypothesis of the strategic use of parasite-poor areas as a way to release birds from the energetic costs on an enhanced immunity (Piersma 1997). In winter, the immune strategy used by nominate godwits was to increase phagocytic activity, which might be relevant for controlling novel and fast replicating pathogens of tropical areas. However, it remains to be answered whether the large variation surrounding soluble components of innate immunity of wintering individuals, were due to the small sample size or if they were reflecting latent infections. Habitat differences between the nominate and the Icelandic subspecies, were also reflected at the cellular level, with nominate godwits having higher

levels of phagocytic cells. This difference could either be explained by an upregulation of innate immunity for the nominate subspecies, or by a lower flexibility of Icelandic birds to upregulate innate response, possibly due to the adrenocortical hormone effects. The adrenocortical hormone is a vital regulator of salt excretion, but may also suppress some immune functions (Gutiérrez et al. 2013). It remains to be tested whether salt regulation hormones cause an overall suppression of the immune system, and if they do so, what implications these effects have in light of the "Piersma (1997) hypothesis". Are birds that downregulate immune responses to reduce energetic costs simultaneously gaining fitness benefits from occupying parasite-poor habitats, or are they unable to upregulate the immune response and therefore becoming more vulnerable in habitats with a higher parasite pressure? These two questions have different implications, from the evolutionary and conservationist points-of-view.

## Chapter II and III: Pathogens maintain MHC gene diversity of adaptive immune defence

For an integrative approach of immunity and immune system strategies, I also explored differences in adaptive immunity between the two godwit subspecies, specifically those involved in the recognition of intracellular pathogens (*e.g.* virus). The MHC class I gene (MHC-I) was chosen due to the vital importance of MHC-I glycoproteins in recognition of intracellular pathogens. MHC characterization studies of Charadriiformes members are vastly underrepresented with only two members studied, and we partly characterize MHC-I gene, exploring in more detail the variability of one of the most polymorphic peptide binding sites of this gene, the exon 3. Characterization was done for the Icelandic godwits, from which I only found classical MHC-I alleles exhibiting the typical characteristics of a functional antigen-presenting gene. Icelandic godwits presented between one and four loci, with at least three of them being expressed. The gene organization of classical loci for the Icelandic godwits was quite similar to their closest relative, the Red knots (*Calidris canutus*), when it comes to the presence of full length alleles and alleles with a 3bp deletion, and also the

sharing of motifs in these full length and 3bp deletion alleles. However, despite these similarities in gene organization and MHC diversity, Icelandic godwits presented alleles with lower polymorphism and fewer sites subjected to positive selection, and were in that respect more similar to marine/coastal species Red-billed gull (*Larus scopulinus*). I suspect that such differences between species could be a result of their "life-style" (resident *vs.* migratory), or even the reflection of the migratory strategy, which exposes Icelandic individuals to a lower pathogenic pressure relative to inland freshwater wader species.

To explore the role of pathogens for the diversifying selection of MHC genes, I compared the two godwit subspecies in terms of MHC diversity and polymorphism. I found that nominate and Icelandic godwits had a considerable overlap in terms of number of alleles (and loci) per individual (nominate: 4-7 alleles; Icelandic: 2-7), but that the two subspecies differed significantly in total number of alleles and polymorphisms. The nominate subspecies had a larger number of private alleles and almost the double number of sites under positive selection compared to the Icelandic subspecies. In natural populations (or subspecies) the maintenance of diversity in neutral parts of the genome are affected by non-selective evolutionary forces such as population size and dispersal patterns (Hughes and Yeager 1998; Sommer 2005). Therefore population demography effect could partly explain the low MHC-I diversity in the Icelandic subspecies, because the population size is about one quarter smaller than that of the nominate subspecies (Delany et al. 2009). A recent study by Trimbos et al. (2014) showed that the neutral gene markers of Icelandic godwits are far less diverse. Nonetheless, the differences in pathogen pressure acting on the Icelandic and nominate godwit subspecies are likely to cause the differences in number of sites under positive selection between the two godwit subspecies, and possibly also differences in polymorphism though the latter needs further analyses. The larger number of positively selected sites in the nominate godwits suggests stronger balancing selection, *i.e.* an adaptation to the pathogen-rich habitats that this subspecies occupies. Adaptive immunity plays an important role for recognition and buffers the impact of multiple pathogens, thus providing a long-standing resistance against commonly encountered diseases. In the advent of the ongoing human impact over ecosystems and environmental changes, these information is highly relevant for conservation biology, once genetic diversity allows

individuals to adapt more quickly, and be more flexible to change which has implications on the long-term survival of animal populations.

## Chapter IV: Sexual selection and advertising capacity to fight disease

This chapter focused on how sexual signals were affected by innate immune function, and whether these signals are honest, *i.e.* truly advertise the quality of mates and their capacity to fight-off pathogens. Parasites are thought to influence mating decisions and behaviour, and animals should avoid mating with parasitized individuals, not only to avoid becoming infected (Clayton 1991), but mainly because females seek good quality males in order to pass on their resistance genes to the progeny (Hamilton and Zuk 1982; Hamilton et al.1990). Females also seek the best providers for them and their offspring (Clayton 1991), and because immune response is a physiological trait that competes for the same resources, signal reliability is kept because low-quality males will not be able to pay the costs of exhibiting high quality sexual secondary plumage characters. Most studies have assessed the trade-offs between induced innate and adaptive immunity to test these assumptions, but I took a different approach and tested whether investment on innate immunity could be reflected by the highly variable melanin-based sexual characters of godwits. These trade-offs could be especially relevant because godwits overlap moult and spring migration; therefore, with such energetic constraints it may be particularly difficult to favour investment of nutrients into a colourful breeding plumage. Our results show that some plumage features of male and female godwits were indeed linked to costly soluble parameters of innate immunity, with some at the expense of body condition. However, the honesty of the signal varied between sexes: it was honest for males but not for females, where an investment into a more colourful plumage were not cost-free. Differences on the honesty of the signal may be related to sex-specific energetic demands and the sex roles of mating behaviour. In times of nutritional stress, besides undertaking migration and moulting, females will bear the costs of egg production and may be unwilling to pay the cost of enhanced breeding plumage at the

expense of lower fecundity and higher vulnerability to disease. On the contrary males are chosen for their bright plumages and for their ability to defend their territory, and our results indicate that females are selecting partners that are not only better at combining moult and migration, but also more responsive against infections.

## Chapter IV: Post sampling effects over the outcome of ecoimmunological assays

Alongside with their simplicity and easy application in studies with free-living and captive birds, the use of multiple immunological assays has become a widespread tool in ecoimmunology studies to comprehensively assess immune variation. However, for logistical and practical reasons, post-sampling collection treatment may differ between studies potentially leading to bias of the final outcome of the assays, and/or not allowing for direct comparisons. One of those post-sampling effects are the unavoidable freeze-thawing cycles that researchers need to undergo when using individual samples for multiple assays. Repeated freeze-thawing effects have proven to affect blood components of humans (Cuhadar et al. 2013), but no information was available for its effects on bird's plasma (or serum) samples. An experimental approach tackling those methodological and logistical effects on the output of these assays was urgently needed. Overall, the results from our experiments are reassuring to researchers, because we proved that the stability of the most commonly tested indices of immune function are mostly unaffected by freeze-thawing cycles, and no substantial variations are introduced by methodological deviations of protocols. This work is therefore a contribution for the fomentation of best laboratory practices, standardization and towards comparable results across different studies.

## Proxy for disease risk: the mosquito-borne pathogens example

Host-pathogen interactions were at the core of this thesis, however this study addressed only the host perspective and did not address the environmental pathogen pressure or give any other information regarding disease prevalence on godwits. The difficulty in taking direct measurements of environmental pathogen pressure starts by defining which type of pathogen we should focus on. Similarly, the complexity of the immune system, comprising all the immunologically-relevant organisms (parasites, commensal or symbionts) shaping immune defence and those that exert higher selective pressures in any study system is a daunting task. The simplest way, would be to focus our attention in a single pathogen and relate the intensity of infection with the activity of the immune system. By doing this we might choose the "wrong parasite" with negligible or even no fitness costs, especially if there are important annual differences in parasite pressure. An alternative way would be to take a more broadly and integrative approach, and use a proxy for infection risk. Considering my own study system and previous studies (*e.g.* Figuerola and Munoz 1999; Mendes et al. 2005; Yohannes et al. 2009; Horrocks 2012; Santos et al. 2012; Pardal et al. 2014; Clark et al. 2015) focused on the freshwater-coastal areas habitat dichotomy and latitudinal effects, these ecological differences are very important for mosquito-borne pathogens and for the outcome of host-parasite interactions. Rainfall and temperature are the main factors regulating vector populations in temperate and tropical areas (Altizer et al. 2006), likewise, the availability of suitable breeding habitats also explains their dependence on freshwater surfaces (Service 2012). Thus, when it comes to environmental disease risk evaluation, I choose to focus my attention on mosquito-borne infections as a proxy measure for pathogen pressure. Outside the scope of the work presented here, on all study sites in which I collected blood samples from godwits, I performed a parallel and systematic vector sampling of mosquitoes (Fig 6, 7) to assess their abundance and competence as transmitters of relevant parasites. Table 1 shows a clear latitudinal gradient in terms of mosquito abundance. Abundance of vectors is not directly translated into actual mosquito-borne infections, because we also need competent ornithophilic (with a clear preference for bird hosts) mosquito species. However, preliminary data indicates that at lower latitudes hosts encounter not only more mosquitoes in the environment, but also a higher diversity of vector species. Hence, for our study species,

the proxy for mosquito-borne infection risk is higher for Guinea-Bissau and Iberia, very low in The Netherlands and non-inexistent in Iceland.

Due to their worldwide distribution (Lapointe et al. 2012) avian malaria and other haemosporidians are frequently used as model system to investigate host-parasite interactions and co-evolutionary processes (Ricklefs et al. 2004). Unlike passerines, waders seem to have rather low blood infection rates (MalAvi database version 2.2.9, December 2017; Bensch et al. 2009). Background data from wader species migrating through the Iberian peninsula, revealed low blood infection rates (Pardal et al. 2014), and no blood infections were found on wintering areas like Guinea-Bissau (unpublished data). However, it is likely that these values are underestimates as the majority of birds screened were species restricted to the rather "clean" coastal areas (*e.g. Tringa totanus*, Redshanks; *Pluvialis squatarola*, Grey Plover), while for waders caught in freshwater habitats the number of screened individuals is low to be conclusive (godwits, N=9; this study). This common and widespread notion that waders are less likely to suffer from haemosporidian infections can be attributed to evolutionary (host-parasite assemblage; Bennett et al. 1993, 1994) and/or ecological/environmental (absence of vectors for transmission of pathogens; Bennett et al. 1992) factors. Only two studies focused on freshwater inland wader species (Mendes et al. 2013; Pardal et al. 2014), and only one presented information on haemosporidians infection rates from waders caught in Africa. In this study the parasite prevalence of freshwater species varied between 6-29% (Mendes et al. 2005), which is similar to that obtained for migratory passerines occupying similar habitats (*e.g. Acrocephalus arundinaceus*, Great reed warbler and *Acrocephalus scirpaceus*, Common reed warbler; Waldenström et al. 2002). Overall, it seems unlikely that waders are not exposed to malaria infections when on their African grounds, but there is a lack of information for tropical areas, and most individuals may have chronic infections, when the parasite is no longer detected in the blood (Mendes et al. 2013). Consequently, our future steps will rely on the identification of important mosquito species for the establishment of relevant host-pathogen interactions (like avian malaria) on our study system, and thus perform a comparative approach that relates the immunological status and MHC-I allele expression levels of the hosts, with the presence of competent and infectious status of the mosquito vectors they encounter on their habitats.

**Table 1.** Mosquito abundance preliminary data from parallel and systematic sampling along the flyway of Black-tailed godwits (*Limosa limosa*). Vectors were collected on the study sites with the use of CDC light traps, which attracts mostly female adult mosquitoes with UV black lights. For trapping host-seeking mosquitoes and turn traps more efficient, $CO_2$ (simulated with dry ice) was used as bait. Sampling effort (Effort) was calculated by multiplying the number of studies areas (N Areas), days of sampling (N Days) and number of traps (N CDC) deployed. The number of mosquitoes in relation to effort (N Mosq/Eff) in bold, represents the total number of mosquitoes caught (N Mosq) corrected for the sampling effort. Mosquito sampling was done covering the full annual-cycle of nominate Black-tailed godwits: Breeding (May-June), Autumn migration (July-September), Wintering (November-December) and Spring migration (January-March); For the Icelandic Black-tailed godwits, sampling covered both the Breeding (June-July) and Wintering (July-March) seasons.

| | **Iceland** | **The Netherlands** | **Portugal** | | | **Guinea-Bissau** |
|---|---|---|---|---|---|---|
| Year | 2013 | 2014 | 2008-2009 | 2008 | 2008-2009 | 2015 |
| Habitat | Marshes | Grassland | Rice-fields & Saltpans | | | Rice-fields |
| Nom. Annual-cycle | - | *Breeding* | *Autumn mig.* | *Wintering* | *Spring mig.* | *Wintering* |
| Icel. Annual-cycle | *Breeding* | - | *W i n t e r i n g* | | | - |
| N Areas | 1 | 1 | 12 | 9 | 9 | 4 |
| N Days | 10 | 11 | 23 | 16 | 24 | 6 |
| N CDC | 3 | 2 | 3 | 3 | 3 | 2 |
| $CO_2$ baited | No | No | Yes | Yes | Yes | No |
| Effort | 30 | 22 | 828 | 432 | 648 | 48 |
| N Mosq | 0 | 6 | 15590 | 279 | 54 | 768 |
| ♀ Mosquitoes (%) | 0 | Unknown | | 92.4 | | 87.6 |
| N Mosq/Eff | **0** | **0.3** | **18.8** | **0.6** | **0.1** | **16** |
| N diff. species | - | Awaits identification | 6 species; 2 complex | | | Prelim. data; min. 6 diff. sp. |
| References | This study | This study | Personal data; Pardal 2009 | | | This study |

**Fig. 6 –** CDC trap deployed on the marshes of Iceland. Photo by Sara Pardal

**Fig. 7 -** Post-collection sample from the rice-fields of Guinea-Bissau. Photo by Sara Pardal

## The impact of ecological changes over immune defence

European long-distance migrants are in sharp decline and increasingly threatened with extinction (Sanderson et al. 2006), a tendency linked to habitat loss, human disturbance and overexploitation (Kuijper et al. 2006; Sanderson et al. 2006; Zwarts et al. 2009). The godwits are no exception, and the nominate subspecies declined strongly in Europe, especially in their main breeding grounds (Kuijper et al. 2006). In their West African wintering grounds, the land reclamation by the overgrowing human population has been rapidly converting natural wetland areas into agricultural land (Zwarts et al. 2009), thus changing the temporal and spatial dynamics of the resources available for many aquatic birds. Although many natural wetlands were converted to rice-fields, which are of great importance for godwits as feeding grounds, land abandonment, crop protective measures and human disturbance continue to be important threats for godwits foraging and roosting in rice-fields (Kleijn et al. 2010). Such land use changes in conjunction with climate change are also disrupting the dynamics of wildlife diseases and host-pathogen interactions, of which vector-borne pathogens are among the most widely known examples due to their sensitivity to changes in temperature and rainfall (Altizer et al. 2013). Land use changes in Africa have been disrupting mosquito's life cycle, because they no long depend on rainfall, using instead the wet rice paddies throughout the year for breeding (*pers. obs.*; Keiser et al. 2005; Muturi et al. 2009; Ramasamy and Surendran 2012). In addition, in the tropics and in other temperate areas that are becoming increasingly warmer due to climate change (Harvell et al. 2009), higher temperatures not only enhance vector population growth and abundance (Pascual et al. 2006), but also promote higher replication rates of parasites within vectors, like for *e.g. Plasmodium* spp. parasites (*i.e.* malaria) (Noden et al. 1995; Altizer et al. 2006). These environmental changes were shown to consistently alter the intensity and/or the distribution range of malaria parasites (Garamszegi 2011; Zamora-Vilchis et al. 2012) and other mosquito-borne pathogens such as West Nile Virus (Paz 2015). Godwits are highly dependent on freshwater areas, and their feeding behaviour potentially makes them highly vulnerable to mosquito-borne infections.

In Chapter I, I show that immune function has an annual rhythm, and that during migration period godwits are significantly immunosuppressed when it comes to their capacity to fight-off new pathogens. The immunosuppressive effects are most likely caused by the energetically demanding long-distance flights and disappear a few days/weeks after arrival, as soon as birds have time to replenish fat and protein stores on wintering sites. During the wintering season in Guinea-Bissau, our data suggests that godwits do up-regulate some components of the innate immune defence, especially relying on inflammatory mediators. Although costly, this strategy seems to pay-off and should be beneficial when it comes to fitness in areas where pathogen pressure is higher. However, it may only be affordable if animals have enough access to resources to maintain this type of response. If the ecological changes induced by habitat loss and lower resource availability on key-staging and wintering sites continues, the struggle for individuals to refuel may seriously unbalance the energetic investments and physiological trade-offs needed during such stressful periods. Moreover, the increased parasitic pressure and/or shifts in the asynchrony of the transmission of mosquito-borne pathogens in their wintering areas and staging areas alike (*e.g.* Iberia during autumn migration; Pardal 2009), might outweigh the investments on immune defence of the hosts, leading to increased disease vulnerability and/or more severe infections. This is likely to reduce the survival of mostly juveniles and some adults. These effects could be especially detrimental considering that adult survival rates are the main responsible for counteracting the continuous population decline of the nominate subspecies (Kleijn et al. 2010). For some African wintering birds like the Red knots and the Eurasian spoonbill (*Platalea leucorodia*), data shows that the African wintering period is the most critical for survival (Lok et al. 2011; Leyrer et al. 2013). But even if the wintering survival rates are not affected, it is known that birds experiencing harsh-conditions or increased workload/energy expenditure in the non-breeding areas, can suffer from carryover effects over reproductive success, long-term survival and ultimately fitness. These carryover effects were shown in several long- and short-distance migrant bird species such as the American redstart (*Setophaga ruticilla*) (Marra and Holmes 2001; Studds and Marra 2005) and Ipswich sparrows (*Passerculus sandwichensis princeps*) (Dale and Leonard 2011). Hegemann et al. (2015) provided the first evidence that variations in innate immunity of Skylarks (*Alauda arvensis*) could predict adult survival of the next breeding season. Therefore, the immune

function was one of the physiological effects mediating experienced harsh conditions and chances to survive until the next breeding season. Consequently, this and other studies provide valuable information for a comprehensive understanding of host pathogen systems, which are important contributors for population dynamics in this fast-declining species. Knowledge regarding immune-related adjustments, vulnerability to disease and possible outcomes of the impact of pathogens in their wild hosts still remains largely unknown (Hawley and Altizer 2011), and may be crucial when contextualized with the ongoing large scale effects of climate change over migratory movements and ecology of animal diseases.

## Sexual selection and innate immunity

Migratory birds can face adverse climatic conditions and fluctuating habitat quality, and many species undergo pre-nuptial body moult on their winter quarters or during spring migration (Zwarts et al. 1990, Newton 2008). Nominate godwits are known to perform a full pre-nuptial body moult during spring migration, while staging on the Iberian rice-fields (Lourenço 2014), while Icelandic godwits moult in their wintering areas in Portugal and Britain (Gunnarsson et al. 2005). For both subspecies there is considerable individual variation in the extent of moult (*pers. obs.*), and, for nominate godwits, the energy required to complete moult forces individuals to stay an extra 6 days after obtaining the necessary energy reserves to depart on their final leg of the northward migration (Lourenço 2014). In nominate godwits, the energy required to moult body feathers represents about 4% of total energy intake, and the minimum time required for an individual to fuel-up in order to continue migration and finish moult is still 17 days (Lourenço 2014). For most migratory birds, early arrival at the breeding sites ensures the access to better territories, and for godwits an earlier breeding leads to higher reproductive output (Gunnarsson et al. 2005) and survival rates of chicks (Schroeder 2010; Lourenço et al. 2011). In addition, the synchrony on the arrival of previously paired breeding individuals at their breeding grounds is around 3 days, so timing is essential to ensure mate retention and avoiding a costly "divorce" (Gunnarsson et al. 2004), which in a monogamous species may represent a missing opportunity to successfully raise progeny on that breeding season. To arrive early at the

breeding grounds, individuals must be able to leave their wintering and/or staging areas earlier or migrate faster (Drent et al. 2003), which puts individuals under a time and energetic constraint. Based on the trade-off between competing physiological demands and food availability (Sheldon and Verhulst 1996; Zuk and Stoehr 2002), such stressful periods can unbalance the allocation of resources into plumage and the quality and extension of these signals. Hence, differences in plumage ornaments have not only fitness consequences through sexual selection (Schroeder et al. 2009), but also may convey information for females about individual quality as proficient foragers and/or the use of high-quality areas. It also means that a heavily parasitized individual will be less likely to undergo moult, while simultaneously replenishing fat scores and perform a timely migration. For example, heavily infected worm parasitized Bar-tailed godwit (*Limosa lapponica*) individuals, were found to be the poorly ornamented (Piersma et al. 2001). Despite the levels of baseline immunity measured at the time of capture (when chicks were hatching in our case) may not be representative of those experienced during moult, these still indicate that only high-quality males were able to maintain higher resistance levels against infection during moult and an up-regulation through most of the breeding season.

Territorial intrasexual competition intensity is not the same for the two godwit subspecies due to different demographic and environmental (agricultural) factors. The population increase of Icelandic godwits likely originates stronger competition for resources and for high-quality territories, while for the nominate godwits the large availability of suitable breeding areas has relaxed competition (Gunnarsson et al. 2005; Schroeder et al. 2009). Indeed, high-quality habitats are generally occupied by more colourful males in Iceland (Schroeder 2010; J.A. Alves *pers. comm.*), but not in The Netherlands (Schroeder et al. 2009). Nonetheless, the data from Chapter IV indicates that irrespective of the subspecies, males, but not females, were the ones in which the extension of the sexual signal, was indeed positively linked with some components of innate immunity and their capacity to control early infections. We have no information regarding normal baseline values of acute phase proteins, like haptoglobin, for our study-species, but the higher levels of this protein potentially indicate that more ornamented individuals (*i.e.* larger colour extent) are likely more prone to suffer injuries during territorial displays and defence, than less ornamented

individuals. Injuries cause inflammation, and the release of haptoglobin offers protection against oxidative stress of the immune response and limits pathogen growth (Juul-Madsen et al. 2008). Likewise, natural antibodies play a critical role for an early efficient neutralization of infections that have reached the blood, limiting the spread to vital organs and a more costly activation of the complement (Ochsenbein and Zinkernagel 2000). The higher levels of these antibodies therefore provide a stronger buffer against larger doses of infectious agents, and these spontaneous baseline levels have a genetic basis (Gobert et al. 1988) and are consequently heritable.

In chapter I, I saw that the status of the innate immune function of both subspecies during pre-nuptial moult period was fairly different, with nominate godwits being relatively immunosuppressed during spring migration, while there were no overall changes on immunity for the Icelandic godwits during winter. For both subspecies, the distances to reach their breeding grounds are fairly similar (Alves et al. 2012; Lourenço 2014), and likewise they both seem to face comparable nutritional bottlenecks, since both occupy areas which are considered to be of high-quality during winter and spring-migration (Alves et al. 2013; Martins et al. 2013). In addition, differential energetic costs of moulting into breeding plumage cannot be attributed to the fact that they rely on different food sources (granivorous *vs.* carnivorous diet) during moult (Schroeder 2010; Viegas et al. 2017). If investments into plumage and immunity are primarily mediated by the availability of resources and temporal bottlenecks, and taking into account that both subspecies start from very different baseline innate immunity values during body moult, the maintenance of an "honest signal" is likely to be energetically less demanding for the Icelandic godwits, than for the nominate ones. For several subspecies of Red knots, it is hypothesized that the energetic investments into breeding plumage are a traded-off against the time and the distance that the birds need to cover to reach their breeding grounds. The lack of available resources to fulfil both physiological demands, are thought to be one of the mechanisms behind the wide variation in the extension and colour intensity of breeding ornaments. The darker and more ornamented subspecies (*Calidris canutus islandica*) shows the least investment to reach their breeding grounds (*i.e.* being closest to breeding areas) (Buehler and Piersma 2008). For both godwit subspecies, if time and nutritional bottlenecks are similar, we should expect a

consistency of breeding plumage colour and extension between them, but interestingly these are not the same. Icelandic godwits have in fact a more exaggerate and darker breeding plumage, while the nominate is less ornamented and paler (Schroeder et al. 2009). The more exuberant breeding plumages of Icelandic godwits can thus be explained since, unlike the nominate godwits, they do not need to invest similar amounts of energy to reach the same immune threshold and keep the honesty of the sexual signal. Considering the energetic balance necessary to migrate, moult and fight-off pathogens, and since the first factors are similar, the subspecies with higher baseline values of immune capacity is the one in advantage. Undoubtfully, this potential explanation of the operating evolutionary mechanisms needs further testing, and it would be interesting to test if the same pattern is found on other branches of immunity, for example the one providing long-lasting immunity (*e.g.* antibody-mediated immunity), and whether if it can be applied to other long-distance wader species.

## Future strategies to tackle the black-box of immune resistance

Throughout the thesis I have used the proxy "better resistance to infection" as individuals that are capable of delivering a higher innate immunity response, an assumption based on the selective pressures of infectious pathogens and antagonistic co-evolution that led to a more effective response of hosts (Baucom and de Roode 2011). However, due to the extreme complexity of the immune system, the ability to control one type of pathogen is not equally correlated to the same efficiency in controlling other infections. Godwits can thus be less susceptible to viral infections (*e.g*. avian influenza; Friend and Franson 1999), but still highly susceptible to other pathogens (*e.g.* parasitic infections), controlled by other immune mechanisms, such as the antibody-mediated immunity (Murphy and Weaver 2017) that was not addressed in our study. Moreover, many authors suggested the existence of trade-offs between the different branches of the immune system (Graham 2001, 2002, 2008), which might underline that a differential investment to produce strong responses in innate immunity, cell-mediated immunity and antibody-mediated immunity, may depend on the type of strategy used to control a specific pathogen (Adamo 2004). This points to the capacity

that the immune system has to reconfigure itself and optimize defence, depending on the threats and physiological trade-offs. In Chapter I, I showed that the innate immunity strategy applied varied dependent on the godwit life-cycle, which is an example that even within the same branch of immunity these trade-offs occur. To really examine these trade-offs, and evaluate the overall disease resistance along the annual cycle of free-living animals, the next logical step should be to evaluate induced innate (*e.g*. delayed hypersensitivity) and antibody mediated humoral adaptive and induced responses. This would require screening of MHC-II genes and experimentally immune challenge birds with novel antigens and measure the strength of such responses (*i.e*. antibody levels) (Norris and Evans2000; Murphy and Weaver 2017). Likewise, determination of the expression levels of both MHC-I and MHC-II would be needed, since I only tackled on Chapter II and III, the potential of these genes to recognize different parasites, when, ultimately, the information encoded needs to be translated into RNA or proteins to ultimately confer protection. For this question, recently developed technologies like high-throughput sequencing and transcriptomics have proved to be valuable tools (Wang et al. 2009; Reuter et al. 2015). These techniques allow researchers to widen the window from the individual to the population level, and to non-model organisms to identify the numerous immunocompetence operating genes, and identify which genes are differentially expressed and specifically measure their expression during immune activation and pathogen infection (*e.g.* Videvall et al. 2015). Another interesting avenue would be to see if the expression levels of certain MHC alleles change on a local or temporal scale in response to pathogen load variations or exposure, and what are the selective advantages of those specific alleles.

Cytokines are also an exciting line for Ecoimmunology research. Cytokines are a large family of small proteins which are key signal molecules for cell communication, movement and proliferation and are important regulators of both innate and adaptive immune responses (Murphy and Weaver 2017). They are also involved in within-host mediation of immune processes (Murphy and Weaver 2017) and are especially important for the dynamics of the immune response towards coinfection of multiple pathogens (*e.g.* Graham 2008). In nature, coinfection is more a rule than an exception and these complex interactions of both pathogens and the immune response have an important influence over disease

outcome. Cytokine roles are rather conservative regardless of the numerous combinations of host-pathogen interactions (Kourilsky and Truffa-Bachi 2001), and should allow us to raise predictions of those interactions on wildlife populations and hence be a useful tool for future studies of Ecoimmunology (Bradley and Jackson 2008).

Another intriguing possibility is the capacity of an individual to tolerate, by minimizing the damage caused by infection (Råberg et al. 2007), rather than resisting a pathogen infection. Tolerance is a different type of defence against parasites, which in mice and many species of plants is a mechanism negatively correlated with resistance to disease (Råberg et al. 2007). In our study system this would be translated as individuals showing a lower immune response, but instead being better able to limit the severity of the infection. To explore this exciting possibility, it would require examination of host genotypes, and relate these to the effects of different parasite intensities, to see which one is more tolerant to the damage caused (*i.e*. typical symptoms) by disease.

To test the ideas about the underlying costs of immunity and resource allocation, and eliminate the effects of confounding factors, an experimental approach would be needed. As an estimation of the cost of deployment an immune response (innate or adaptive), researchers would need to perform controlled infections on captive birds and measure basal metabolic rates (BMR) changes (Lochmiller and Deerenberg 2000). Because costs of deployment can only be visible under energetic limited environments, experimental design should include blocks with birds under a control access to food and/or under different temperature regimes. This approach for measuring the costs of defence have been rarely applied to free-living birds. The few exceptions, estimated the effects of deployment an induced innate (Hegemann et al. 2012b; Hegemann et al. 2013b) and adaptive humoral defence (Mendes et al. 2006b; Abad-Gómez et al. 2013) over basal metabolic rates (BMR), but to the best of my knowledge only Gutiérrez et al. (2011b) has measured the costs of immune deployment under a constrained energetic environment. Such experiments would be valuable to validate or raise other ecoimmunological hypotheses, concerning the costs of immune responses.

Ecoimmunological studies face many practical challenges due to the difficulties of validating hypotheses about the drivers of immune variations in free-living birds. Likewise, the immune system and the host-pathogen ecological systems are very complex. Nonetheless, this new field of research is crucial for understanding disease susceptibility variation in wild populations in relation to deteriorating environmental conditions, and ultimately how these changes affect the long term survival and fitness of many bird species.

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