Exploring Endoparasites and Physiological Stress: Insights from a European Forest Bird Community

Dissertation

“kumulativ”

Zur Erlangung des Grades eines

Doktor der Naturwissenschaften

(Dr. rer. nat.)

am Fachbereich Biologie der Philipps-Universität Marburg

Vorgelegt von

Finja Strehmann

aus München

Marburg, April, 2024

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Illustration auf der Titelseite: Kohlmeise (*Parus major*) und Rotkehlchen (*Erithacus rubecula*) in einem Waldökosystem. Das Bild wurde von Finja Strehmann angefertigt.

Die vorliegende Dissertation wurde von April 2021 bis März 2024 am Fachbereich Biologie der Philipps-Universität Marburg unter Leitung von Prof. Dr. Nina Farwig angefertigt.

Vom Fachbereich Biologie der Philipps-Universität Marburg (Hochschulkennziffer 1180) als Dissertation angenommen am 15.04.2024.

Erstgutachter(in): Prof. Dr. Nina Farwig

Zweitgutachter(in): Dr. Sascha Rösner

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Table of content

Declaration of the author contributions

The thesis "The influence of environmental factors on stress and parasites in a forest bird community" is based on the work I carried out from April 2021 to March 2024 at the University of Marburg, under the supervision of Prof. Dr. Nina Farwig and Dr. Sascha Rösner. The chapters II to V of this thesis consist of four independent scientific manuscripts, each with co-authorship, and have been or will be published in peer-reviewed journals. The contributions of the authors for each manuscript are stated as follows:

Chapter II – Half of a forest bird community infected with haemosporidian parasites

Finja Strehmann, Marcel Becker, Kim Lindner, Juan F. Masello, Petra Quillfeldt, Yvonne R. Schumm, Nina Farwig, Dana G. Schabo, Sascha Rösner

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FS contributed to the conception and design of the study, carried out the field and laboratory work, performed the statistical analyses, and wrote the manuscript. NF, DS, and SR designed and structured the study. PQ, JM, and YS helped with conceptual and analytical approaches and supported the lab and fieldwork. MB, KL, DS, and SR contributed to fieldwork. YS introduced and helped with both field and laboratory work. NF, DS, and SR contributed to the statistical analysis. All authors contributed to manuscript revision.

Chapter III – Identifying and Counting Avian Blood Cells in Whole Slide Images via Deep Learning

Markus Vogelbacher, **Finja Strehmann**, Hicham Bellafkir, Markus Mühling, Nikolaus Korfhage, Daniel Schneider, Sascha Rösner, Dana G. Schabo, Nina Farwig, Bernd Freisleben

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Visualization, MV; Supervision, MM, NF, DGS, SR and BF; Project administration, NF and BF; Funding acquisition, NF and BF; All authors have read and agreed to the published version of the manuscript.

Chapter IV – Intrinsic factors influence physiological stress in a forest bird community: Adults and females have higher H/L ratios than juveniles and males.

Finja Strehmann, Markus Vogelbacher, Clara Guckenbiehl, Yvonne R. Schumm, Juan F. Masello, Petra Quillfeldt, Nikolaus Korfhage, Hicham Bellafkir, Markus Mühling, Bernd Freisleben, Nina Farwig, Dana G. Schabo, Sascha Rösner

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FS contributed to the conception and design of the study, carried out the field and laboratory work, contributed to the conception and development of the neural network approach, performed the statistical analyses, and wrote the manuscript. NF, DS, and SR designed and structured the study and participated in drafting the manuscript. PQ, JM, and YS helped with conceptual and analytical approaches and supported the lab and fieldwork. DS, DG, and SR contributed to fieldwork; YS introduced and helped with both field and laboratory work; MV, MM, NK, HB and BF contributed to the conception and development of the neural network approach, NF, DS, CG and SR contributed to the statistical analysis. All authors contributed to manuscript and read and approved the submitted version.

Chapter V – Birds infected with blood parasites react differently to their environment

Finja Strehmann, Yvonne R. Schumm, Juan F. Masello, Petra Quillfeldt, Nina Farwig, Dana G. Schabo, Sascha Rösner

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and structured the study. PQ, JM, and YS helped with conceptual and analytical approaches and supported the lab and fieldwork. DS, and SR contributed to fieldwork. YS introduced and helped with both field and laboratory work. NF, DS, and SR contributed to the statistical analysis. All authors contributed to manuscript revision and have read and approved the submitted version.

Summary

The global biodiversity crisis is advancing steadily. Additionally, climate change increases the risk of emerging infectious diseases (EIDs). Due to their global migration, birds are of special interest when studying the spread of pathogens and parasites. Avian influenza, *Trichomonas gallinae*, and the Usutu virus are only some examples of EIDs that had significant negative effects on their host species populations. However, also more less prominent parasites and pathogens are expected to shift their distribution ranges in response to climate change, such as avian malaria and other haemosporidian parasites. While infection with haemosporidian parasites is usually not lethal in co-evolved host species, host-switching events have had severe negative effects on the new host species, even leading to local extinctions of some species.

Given their importance in understanding emerging infectious diseases (EIDs) and documented population declines linked to environmental factors, birds play a crucial role in studying the impact of pathogens, parasites, and environmental changes on populations and communities. While population declines are often only recognized when the number of individuals decreases, physiological measures offer the potential to predict declines and identify their causes. For instance, assessing stress levels in birds, which is associated with fitness loss, can serve as an early warning indicator, particularly when combined with monitoring for parasites. One method for assessing the physiological stress response in birds is by using the ratio of heterophils to lymphocytes (H/L ratio) which has been found to increase in response to increased stress hormones.

Both physiological stress and the effects of parasites have primarily been studied within single species. However, there is a lack of research on bird communities. In my doctoral thesis, I delved deeper into stress and endoparasites within a European forest bird community. My aim was to determine the suitability of analysing these factors as warning indicators across multiple species and to assess the influence of environmental factors on them. To achieve this, I studied the prevalence, diversity, and host-parasite interactions of endoparasites and their hosts within the community. Additionally, I contributed to the development of a novel deep learning approach for automatic avian blood cell identification and counting. Furthermore, I evaluated the natural variation of the H/L ratio across the bird community, considering factors

such as phylogeny, sex, age, body condition, and incubation status. Lastly, I investigated the impact of temperature, precipitation, and shrub layer, as well as endoparasite infection, on the H/L ratio of the forest bird community.

For data collection, I captured and sampled 483 bird individuals belonging to 29 species in a managed forest in Central Germany over four breeding seasons. I collected saliva and blood samples to examine infections with endoparasites, with a focus on *T. gallinae* and haemosporidian blood parasites. Additionally, the blood samples were used to determine the H/L ratio from a blood smear.

In my research, I found that nearly half of the sampled birds (48.1%) were infected with blood parasites. However, these infections were not evenly distributed across species; some species, such as *Parus major* and *Turdus merula*, exhibited significantly higher susceptibility to infections compared to others. For instance, no infections were found in species such as *Certhia familiaris* and *Dendrocopos major*.

To improve blood smear analysis and ensure consistent counting, I contributed to developing a deep learning method for identifying and counting bird blood cells. It involved two neural networks: one for selecting countable regions with a 97.3% F1 score (the harmonic mean of precision and recall), and another for identifying and counting cells within these regions, achieving a mean average precision of 90.7%.

Based on the resulting count data I calculated the H/L ratio and detected a strong phylogenetic signal across the studied forest bird community. I further found general patterns of the influence of intrinsic factors on the H/L ratio within the community. Females had significantly higher H/L ratios than males and adult birds had higher H/L ratios than females and adults had higher H/L ratios than juveniles. We further found that birds involved in the incubation of eggs tended to have higher H/L ratios than non-incubating birds. Body condition did not significantly affect the H/L ratio across the bird community. These findings emphasise the importance of controlling for intrinsic factors when using the H/L ratio as an indicator.

Finally, I also included abiotic factors (temperature and precipitation) and biotic factors (shrub layer) in my analyses and found that higher mean decade temperature had a significant negative impact on the H/L ratio. The results further indicate that infected birds respond differently to environmental factors than uninfected ones. The stress level in infected birds

does not change with temperature, and while uninfected birds tended to be less stressed at capturing sites with denser shrub layer, infected birds show contrasting trends.

Based on my analyses, I demonstrated that both stress and parasite infestation can be evaluated across species in a forest bird community and hence potentially serve as early warning indicators for bird communities especially since both factors have been associated with reduced reproductive success and reduced survival in previous studies. However, future studies in bird communities should also incorporate additional factors such as flight activity and reproductive success to confirm the validity of stress and parasite infestation as early warning indicators.

Zusammenfassung

Die weltweite Biodiiversitätskrise schreitet stetig voran, wobei Klimawandel, Landnutzungsänderungen, Umweltverschmutzung, die Ausbeutung natürlicher Ressourcen und die Einführung invasiver gebietsfremder Arten die wichtigsten Treiber sind. Außerdem erhöht der Klimawandel das Risiko neu auftretender Infektionskrankheiten (EIDs). Aufgrund ihres globalen Migrationsverhaltens sind Vögel von besonderem Interesse für die Untersuchung der Verbreitung von Krankheitserregern und Parasiten. Vogelgrippe, *Trichomonas gallinae* und das Usutu-Virus sind nur einige Beispiele für EIDs, die erhebliche negative Auswirkungen auf die Populationen ihrer Wirtsarten hatten. Es ist jedoch davon auszugehen, dass auch weniger bekannte Parasiten und Krankheitserreger ihre Verbreitungsgebiete als Reaktion auf den Klimawandel verlagern werden, wie etwa Vogelmalaria und andere Blutparasiten der Ordnung Haemosporida. Während eine Infektion mit Blutparasiten bei koevolvierten Wirtsarten in der Regel nicht tödlich verläuft, kann ein Wirtswechsel schwerwiegende negative Auswirkungen auf die neuen Wirtsarten haben und sogar zum lokalen Aussterben einiger Arten führen.

Angesichts ihrer Bedeutung für das Verständnis neu auftretender Infektionskrankheiten (EIDs) und des dokumentierten Rückgangs von Populationen auf Grund von Umweltveränderungen spielen Vögel eine entscheidende Rolle bei der Untersuchung der Auswirkungen von Krankheitserregern, Parasiten und Umweltveränderungen auf Populationen und Gemeinschaften. Während Populationsrückgänge oft erst dann erkannt werden, wenn die Zahl der Individuen abnimmt, bieten physiologische Maße die Möglichkeit, Rückgänge früher zu erkennen und ihre Ursachen zu ermitteln. So können beispielsweise erfasste Stressantworten bei Vögeln, welche mit einem Fitnessverlust einhergehen können, als Frühwarnindikator dienen, insbesondere in Kombination mit einem Parasiten Monitoring. Eine Methode zur Bewertung der physiologischen Stressreaktion bei Vögeln ist die Verwendung des Verhältnisses von Heterophilen zu Lymphozyten (H/L-Verhältnis), das nachweislich als Reaktion auf erhöhte Stresshormone ansteigt.

Sowohl der physiologische Stress als auch die Auswirkungen von Parasiten wurden bisher hauptsächlich bei einzelnen Arten untersucht. Es mangelt jedoch an Untersuchungen von gesamten Vogelgemeinschaften. In meiner Doktorarbeit habe ich mich eingehender mit Stress und Endoparasiten in einer europäischen Waldvogelgemeinschaft beschäftigt. Mein Ziel war es, die Eignung der Analyse dieser Faktoren über mehrere Arten hinweg zu ermitteln und den Einfluss von Umweltfaktoren auf diese Faktoren zu bewerten. Zu diesem Zweck habe ich die Prävalenz, die Vielfalt und die Wirt-Parasiten-Interaktionen von Endoparasiten und ihren Wirten innerhalb der Vogelgemeinschaft untersucht. Außerdem trug ich zur Entwicklung eines neuartigen Deep-Learning-Ansatzes für die automatische Identifizierung und Zählung von Blutzellen bei Vögeln bei. Darüber hinaus bewertete ich die natürliche Variation des H/L-Verhältnisses in der Vogelgemeinschaft unter Berücksichtigung von intrinsischen Faktoren wie Phylogenie, Geschlecht, Alter, Körperzustand und Inkubationsstatus. Schließlich untersuchte ich die Auswirkungen von Temperatur, Niederschlag und Strauchschicht sowie von Endoparasitenbefall auf das H/L-Verhältnis der Waldvogelgemeinschaft. Im Rahmen der Datenerhebung habe ich 483 Vogelindividuen aus 29 Arten in einem bewirtschafteten Wald in Mitteleuropa über vier aufeinanderfolgende Brutzeiten erfasst und beprobt. Ich sammelte Speichel- und Blutproben, um Infektionen mit Endoparasiten zu untersuchen, wobei der Schwerpunkt auf *T. gallinae* und Blutparasiten der Ordnung Haemosporida lag. Darüber hinaus wurden die Blutproben auch verwendet um einen Blutausstrich zu machen und das H/L-Verhältnis zu bestimmen.

Im Rahmen Forschung fand ich heraus, dass fast die Hälfte der beprobten Vögel (48,1%) mit Blutparasiten infiziert war. Diese Infektionen waren jedoch nicht gleichmäßig über die Arten verteilt; einige Arten, wie *Parus major* und *Turdus merula*, wiesen im Vergleich zu anderen signifikant höhere Anfälligkeiten für Infektionen auf. Andererseits wurden bei Arten wie *Certhia familiaris* und *Dendrocopos major* keine Infektionen festgestellt.

Des Weiteren trug ich zur Entwicklung einer Deep-Learning-Methode zur Identifizierung und Zählung von Vogelblutzellen bei um die Analyse von Blutausstrichen zu verbessern und eine konsistente Zählung sicherzustellen. Es handelte sich um zwei neuronale Netze: eines zur Auswahl zählbarer Regionen welches einen F1-Score von 97,3% erreichte und ein weiteres zur Identifizierung und Zählung von Zellen innerhalb dieser Regionen, welches eine durchschnittlichen Präzision von 90,7% hat.

Basierend auf den resultierenden Zähldaten berechnete ich das H/L-Verhältnis und stellte ein starkes phylogenetisches Signal desselben innerhalb der untersuchten Waldvogelgemeinschaft fest. Darüber hinaus fand ich allgemeine Muster des Einflusses intrinsischer Faktoren auf das H/L-Verhältnis innerhalb der Gemeinschaft. Weibchen hatten signifikant höhere H/L-Verhältnisse als Männchen, und adulte Vögel hatten höhere H/L-Verhältnisse als Jungvögel. Darüber hinaus stellten wir fest, dass Vögel, die an der Inkubation von Eiern beteiligt waren, tendenziell höhere H/L-Verhältnisse aufwiesen als nicht brütende Vögel. Die körperliche Kondition hatte keinen signifikanten Einfluss auf das H/L-Verhältnis innerhalb der Vogelgemeinschaft. Diese Ergebnisse betonen die Bedeutung der Kontrolle intrinsischer Faktoren bei der Verwendung des H/L-Verhältnisses als Indikator.

Schließlich habe ich auch abiotische Faktoren (Temperatur und Niederschlag) und biotische Faktoren (Strauchschicht) in meine Analysen einbezogen und festgestellt, dass Dekaden mit einer höheren mittleren Temperatur einen signifikant negativen Einfluss H/L-Verhältnisse als hatten. Die Ergebnisse deuten weiterhin darauf hin, dass mit Blutparasiten infizierte Vögel anders auf Umweltfaktoren reagieren als nicht infizierte Vögel. Das H/L-Verhältnis bei infizierten Vögeln ändert sich nicht mit der Temperatur, und während nicht infizierte Vögel dazu neigten, an Standorten mit dichterer Strauchschicht weniger gestresst zu sein, zeigten infizierte Vögel gegensätzliche Trends.

Basierend auf meinen Analysen habe ich gezeigt, dass sowohl Stress als auch Parasitenbefall in einer Waldvogelgemeinschaft über mehrere Arten hinweg bewertet werden können und daher potenziell als Frühwarnindikatoren für Vogelgemeinschaften dienen können, insbesondere da beide Faktoren mit reduziertem reproduktiven Erfolg und reduziertem Überleben in Verbindung gebracht wurden. Zukünftige Studien in Vogelgemeinschaften sollten jedoch auch zusätzliche Faktoren wie Flugaktivität und reproduktiven Erfolg einbeziehen, um die Nutzbarkeit von Stress und Parasitenbefall als Frühwarnindikatoren zu bestätigen.

Chapter I: General introduction

The global biodiversity crisis is advancing steadily with climate change, land use change, pollution, the exploitation of natural resources, and the introduction of invasive alien species being the most significant drivers (IPBES 2019; Jaureguiberry et al. 2022). These drivers decrease both the survival probability and the reproductive success of individuals and consequently lead to negative population trends (Leech und Crick 2007; Grazer und Martin 2012). While efforts of the United Nations, its member states, and the European Union, intended slowing or halting the loss of biodiversity, 25% of all recorded animal and plant groups still remain endangered (European Commission 2011; IPBES 2019; CBD 2022).

Alongside these ongoing conservation challenges, the spread of emerging infectious diseases (EIDs) is accelerated by globalisation and climate change (Daszak et al. 2000; Cunningham et al. 2017). While pathogens and parasites in general are important drivers of ecological and evolutionary processes, the accelerated spread of EIDs poses a major threat to wildlife populations and communities as well as human health (Daszak et al. 2000; Jones et al. 2008; Fuller et al. 2012; Gómez und Nichols 2013; Okamura et al. 2018; Lymbery und Smit 2023). The effects of the environment on disease dynamics underscore the interconnectedness of healthy ecosystems, the well-being of both wild and domestic animals, and human health, which has been widely recognised and resulted in the One Health approach (One Wold - One Health 2004; FAO et al. 2008; Cunningham et al. 2017). In the One Health approach governments, academia, and civil society join forces to address current issues such as pathogen spill over, climate change and antimicrobial resistances (Gruetzmacher et al. 2021). Especially after the COVID-19 outbreak which was predicted however not prevented, the importance of the One Health approach was further emphasised (Gruetzmacher et al. 2021). In the past centuries, outbreaks of infectious diseases have been observed in various species including plants, invertebrates and vertebrates. Some recent examples for such diseases are *Xylella fastidiosa*, a bacterium which is transmitted by sap sucking insects and infects at least 595 different plant species (Trkulja et al. 2022), the crayfish plague (*Aphanomyces astaci*) which caused a significant loss of native crayfish populations after being introduced to Europe (Jussila et al. 2021) and *Batrachochytrium salamandrivorans* which is responsible for the decline of amphibian populations (Lastra González et al. 2019). One of the most prominent

EIDs is avian influenza which caused mass mortality events in wild birds and had a severe impact on the poultry industry (Adlhoch et al. 2022; Adlhoch et al. 2023).

Birds in general are highly susceptible to EIDs and of special interest when studying EIDs due to their migratory behaviour across large geographical distances (Fuller et al. 2012). Other than avian influenza, birds are hosts to a large variety of EIDs such as the Usutu virus, the West Nile Virus and *Trichomonas gallinae* which have led to drastic population declines in their host species (Kilpatrick et al. 2006; Peters et al. 2009; Fuller et al. 2012; Peters und Ludwichowski 2014; Lühken et al. 2017; Michel et al. 2019; Störk et al. 2021). In combination with climate change other, less prominent parasites and pathogens, such as avian malaria, are expected to shift their distribution ranges and infect new host species in the future (Valkiūnas 2005; Garamszegi 2011; Fuller et al. 2012). Avian malaria and related species of the order haemosporida are almost globally distributed, transmitted by vectors and have long co-evolved with their avian hosts (Baker 1967; Gylstorff und Grimm 1987; Valkiūnas 2005; Bensch et al. 2009; Santiago-Alarcon et al. 2012; Clark et al. 2014; Lauron et al. 2015). Even though the infection severity is typically low in established host-parasite interactions, incidents from Hawaii have demonstrated that the introduction of vectors and parasites into new areas and the infection of new host species, can have strong negative effects on novel host populations, and have even led to the local extinction of some species, as for example the Hawaii Oo (*Moho nobilis*) or the Lanai Creeper (*Paroreomyza montana newtoni*; van Riper et al. 1986; Atkinson und van Riper 1991; Atkinson et al. 1995; Atkinson et al. 2000; Atkinson und LaPointe 2009). Considering the possibility of extinction events, the still existing knowledge gaps on distribution ranges of haemosporidian species and their genetic lineages, along with their potential host-parasite interaction partners, may be problematic (Fuller et al. 2012; Dunn et al. 2023). Shifts in global distribution patterns and host-shifts may occur unnoticed without comprehensive knowledge of these factors. Therefore, the potential impact of blood parasites on population dynamics may be underestimated, which could lead to misinterpretations and an incomplete understanding of the drivers causing population declines.

Birds can help to understand the impact of climate change and the spread of diseases on wildlife populations and communities due to their key-role in emerging diseases, documented population declines as well as the knowledge of previous population dynamics, (Jetz et al. 2007). Long-term data have revealed a 29% decline in the abundance of bird individuals in

North America (Rosenberg et al. 2019) and in the EU less than half of the bird species (47%) have a good population status (European Environment Agency 2020). The decline in bird abundance has been most pronounced among the most common species (Inger et al. 2015), with farmland and grassland birds being particularly at risk due to land-use change and the intensification of agriculture (European Commission und Directorate-General for Environment 2022; Rigal et al. 2023). The majority of forest species, on the other hand, show a stable or even positive population trend (Gregory et al. 2019; Reif et al. 2022). While generalist species are increasing, forest specialists especially those of the shrub layer are decreasing significantly (Reif et al. 2022; Maag et al. 2024).

To gain a better understanding of population dynamics, it is useful to also monitor the physiological responses of organisms to their changing environment (Wikelski und Cooke 2006; Dantzer et al. 2014; Seebacher et al. 2023). This can help in identifying the causes of population declines and developing suitable conservation tools (Wikelski und Cooke 2006; Seebacher et al. 2023). One physiological measure frequently used in conservation physiology is the stress response (Wikelski und Cooke 2006; Davis et al. 2008; Busch und Hayward 2009). Stress is a physiological response to environmental challenges or disturbances, which includes changes in hormone levels, metabolism, behaviour, and overall health status (Romero 2004; Martin 2014). Although stress response can in general also be beneficial in the short term and enables organisms to adapt to their environment and to cope with unfavourable situations, ongoing exposure to stressors can cause a chronic stress response which has been found to having detrimental effects on health and fitness (Moberg 2000; Harris et al. 2002; Cyr und Romero 2007; Martin 2014). To estimate the effects of environmental stressors on bird populations, it is crucial to understand the underlying physiological adaptations and to know the informative value of the chosen method of stress measure (Wikelski und Cooke 2006; O'Dell et al. 2014; Gormally und Romero 2020). One commonly used method for assessing stress levels is by quantifying glucocorticoid hormones (Sheriff et al. 2011; Davis und Maney 2018). These hormones can be measured in various tissues, including blood, saliva, feathers, and faeces (Sheriff et al. 2011). They provide insights into the current stress status based on endogenous cycles, immediate prior experience, and long-term experience (in the case of blood and saliva), the average stress over a certain species-specific amount of time (in the case of faeces), or the long-term daily stress fluctuations during moult (in the case of feathers; Sheriff et al. 2011). Measuring glucocorticoids from blood, however, requires quick sampling

within three minutes of capture to assess baseline stress rather than stress response to capture (Sheriff et al. 2011; Davis und Maney 2018). Oxidative stress can also be measured in birds and is another frequently applied method by ornithologists to understand their physiological responses to the environment (Davis et al. 2008; Fokidis et al. 2008; Frigerio et al. 2017; Xia und Møller 2018; Dimitrov et al. 2019; Gutiérrez et al. 2019). Unlike glucocorticoids, which reflect acute stress, oxidative stress indicates nutritional stress and overall health (Davis et al. 2008; Fokidis et al. 2008; Frigerio et al. 2017; Xia und Møller 2018; Dimitrov et al. 2019; Gutiérrez et al. 2019). The method I used to assess stress in birds is the ratio of heterophils to lymphocytes (H/L ratio), which are both leukocytes and therefore part of the immune system (Davis et al. 2008). In response to stress, lymphocytes are redistributed to other tissues, while heterophils are released into the bloodstream (Davis et al. 2008). These adaptations are however not as prompt as hormonal changes and typically take 30 to 60 minutes (Davis 2005; Cīrule et al. 2012). The H/L ratio allows for a more flexible sampling scheme than when measuring glucocorticoids when drawing a blood sample within three minutes is not feasible (Davis et al. 2008).

The H/L ratio is commonly employed by ornithologists to evaluate the physiological response of bird species to specific stimuli (Davis et al. 2008). Several studies have shown that the H/L ratio differs between different habitats, and changes in response to temperature changes, high noise, social factors such as pairing, nutritional status as well as parasite infection (Davis et al. 2008; Fokidis et al. 2008; Krams et al. 2011; Bańbura et al. 2013a; Bańbura et al. 2013b; Dunn et al. 2013; Biard et al. 2015; Frigerio et al. 2017; Włodarczyk et al. 2018; Bain et al. 2019; Dimitrov et al. 2019; Ibáñez-Álamo et al. 2020; Ribeiro et al. 2022). Intrinsic factors such as sex and age have been found to cause variation in the H/L ratio in birds, yet studies examining single species have shown a range of outcomes, underscoring the complexity and variability of these relationships (Hõrak et al. 1998; Campo und Davila 2002; Kilgas et al. 2006; Norte et al. 2009; Jakubas et al. 2011; Skwarska et al. 2019; Vincze et al. 2022). As elevated stress levels, including elevated H/L ratios, have been linked to reduced reproductive success, the H/L ratio has the potential to serve as a valuable warning indicator for bird populations., (Hõrak et al. 1998; Arida 2005; Breuner 2011; Strasser und Heath 2013; Calisi et al. 2018).

However, there are still some remaining challenges when using the H/L ratio. Firstly, evaluating blood smears under the microscope and identifying and counting the blood cells

require considerable expertise and time (Ruiz et al. 2002; Davis et al. 2008). Furthermore, so far studies have focussed on single species and due to interspecific variation, the H/L ratio has not been applied to bird communities yet. A study analysing leukocyte profiles of nearly 250 bird species has however found a strong phylogenetic signal in the H/L ratio which allows to account for some of the species-specific differences of this measure (Minias 2019). Another challenge lies in the lack of baseline levels for the H/L ratio due to differences among sexes, ages, and life stages of birds (Davis et al. 2008; Norte et al. 2009; Müller et al. 2010; Jakubas et al. 2011). Moreover, the H/L ratio's association with immunity suggests potential fluctuations in response to factors such as parasites, pathogens, and investment in the immune system (Sanz et al. 2004; Dunn et al. 2013; Krams et al. 2013; Biard et al. 2015; Clark 2015). Nevertheless, when being aware of these influence factors, the H/L ratio can be a useful tool for comparing individuals, populations, and communities as a relative stress measure and has the potential to serve as a tool for identifying environmental stressors.

If and how the H/L ratio can however be used not only for assessing single species but also within a bird community has not been evaluated and nor are the host-parasite interactions of birds and haemosporidian parasites of a managed central European forest bird community. By investigating the host-parasite interactions of a forest bird community, developing a novel tool for the evaluation of avian blood smears, understanding underlying intrinsic fluctuations of the H/L ratio across a bird community and examining the effects of environmental conditions and parasite infection on the H/L ratio, my thesis aims to advance the utilization of the H/L ratio as a tool for conservation physiologists and extend its applicability to community-based studies.

Aims of the thesis

By studying a forest bird community and its endoparasites, I aim to uncover the diversity of endoparasites and host-parasite interactions. Additionally, it focuses on examining the physiological stress response of birds and advancing the methodology for assessing the heterophil to lymphocyte ratio (H/L ratio) through the development of a deep learning approach. By evaluating the applicability of the H/L ratio across a bird community, this study further aims to enhance its applicability as a tool for future conservation physiological assessments and ecological research.

To achieve my objectives, I studied a Central European forest bird community during four consecutive breeding seasons. The study was carried out in the 'Marburg Open Forest' (MOF), a 250-hectare managed mixed deciduous forest in Central Hesse (refer to Figure 1.1). Birds were captured at ten mist-netting sites, which covered the different habitat types (determined by tree composition and vegetation heterogeneity) and were evenly distributed across the MOF (Figure 1.1).

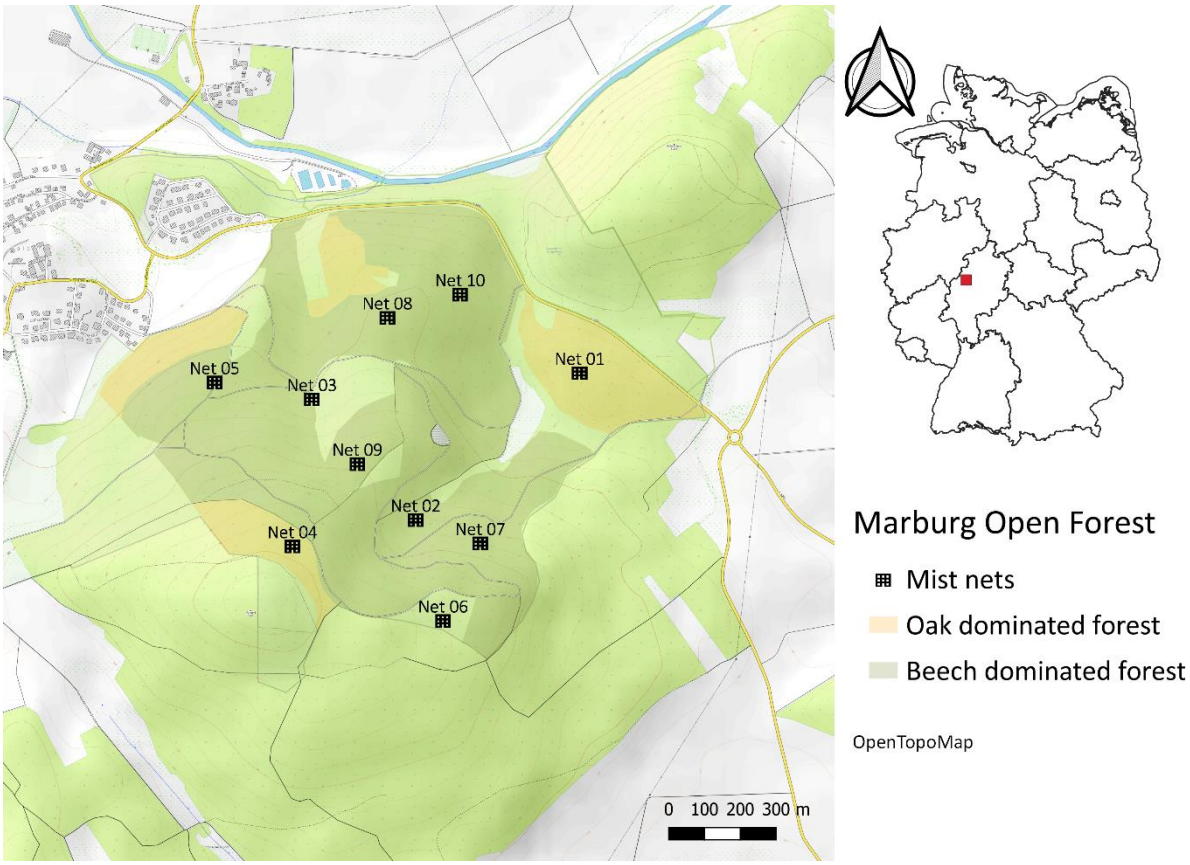

Figure 1.1: Map of the study area showing forest cover (green) and dominant tree species (colour layers). Mist-netting sites are indicated by black nets.

I have structured my work into chapters based on the manuscripts I have authored during the course of my doctoral studies. Following this general introduction, the chapters II to V present the different work packages of my thesis. Due to the cumulative nature of this thesis, each of these four chapters is written in the format of a scientific article and includes the scientific background, material and methods, results as well as a discussion for the results of the respective chapter. While I served as the primary author for chapters II, IV, and V, I utilized the term 'we' in these chapters to acknowledge the contributions and collaborative efforts of

my co-authors. For detailed information about the contribution of each co-author, please refer to the declaration of author contributions (page 1 to 3).

In Chapter II, I analysed which endoparasites are infesting the forest bird community, focusing on *Trichomonas gallinae* and blood parasites of the order Haemosporida. To determine the parasites, I took blood and saliva samples from the birds and analysed them genetically in the laboratory. My aim was to analyse parasite diversity and abundance and to create a host-parasite network. In addition, I wanted to find out whether parasite prevalence differs between bird families, expecting common families with many closely related species to be more frequently infested by parasites. I also expected abundant families to have a higher parasite diversity and families with a high parasite diversity to have a higher parasite prevalence.

In Chapter III, I teamed up with computational scientists to develop a new deep-learning approach aimed at enabling a more precise and effective analysis of blood smears for determining the H/L ratio. To achieve this goal, I first prepared the blood smears of the birds in the laboratory (fixation and staining) and then digitized them using a whole-slide scanner. Based on the resulting images, we developed a two-step neural network. In the first step, the network divides the smears into tiles and categorizes them as countable or uncountable depending on their quality. Subsequently, an instance segmentation model detects the cells and classifies them as erythrocytes or leukocytes, with the leukocytes further subdivided into lymphocytes, heterophils, and eosinophils. This novel approach should allow for the evaluation of a larger area of the smear than traditional methods. Therefore, I expect achieving better and more reliable results using this approach.

In Chapter IV, I explored how intrinsic factors such as sex, age, and reproductive status influence the H/L ratio in a forest bird community. Combining the individual bird metadata with data from the deep learning method introduced in Chapter III, I aimed to uncover species-specific variations and assess the strength of the phylogenetic signal in the H/L ratio. Additionally, I hypothesized that adult birds would exhibit higher H/L ratios than juveniles, females would show higher ratios than males, and actively breeding birds would demonstrate higher ratios than non-breeding individuals.

In Chapter V, my aim was to combine H/L ratio data with information on parasite infections and environmental factors—specifically temperature, precipitation, and shrub layer density—

in order to identify overarching drivers of stress within a forest bird community. In this analysis, I considered both the variation in the H/L ratio explained by phylogenetic relatedness and intrinsic factors. I expected that birds would i) exhibit lower stress levels during warmer temperatures, ii) higher stress levels during periods of high precipitation, and iii) lower H/L ratios in areas with dense shrub layers.

Chapter II: Half of a forest bird community infected with haemosporidian parasites

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2.1 Abstract

Parasites play important roles in ecosystems. Through their interactions with host and vector species, they are capable of changing the behavior and population dynamics of their host species, and the shape of entire communities. Over the past years, many studies have acknowledged the role of parasitism for host populations and communities and discovered their important regulatory functions for many vertebrate populations. Although birds are a well-studied group of vertebrates, the infection patterns of endoparasites at the community level are not fully understood. Some bird species and families are known to have a higher susceptibility to certain endoparasites than others, which may be driven by their abundance in the community.

Over the course of four consecutive breeding seasons (2019–2022), we monitored the patterns of endoparasite infections in a bird community of a temperate forest ecosystem. We sampled 483 birds belonging to 29 Palearctic species and investigated the prevalence of blood parasites (haemosporidian parasites) and Trichomonas spp. using molecular methods.

We found an overall prevalence of 48.1% of haemosporidians belonging to 53 genetic lineages of the three genera Haemoproteus, Leucocytozoon, and Plasmodium spp. While the bird families Turdidae (94%) and Paridae (76%) showed a high prevalence of haemosporidians, Certhiidae, and Picidae were not infected (0%). Host–parasite network analysis detected high variability in interactions. Infections with Trichomonas spp. were not observed in the forest bird community.

We found that the prevalence and lineage diversity of haemosporidian parasites differed between avian families and that the parasite prevalence of a family could not serve as a

predictor of lineage diversity. To further assess the consequences of these host–parasite interactions for bird communities, future research should aim to disentangle the infection pathways in different ecosystems while also considering the vector community and environmental factors.

2.2 Introduction

Parasites play an important role in ecosystems and are known for their ability to influence population dynamics and shape communities (Gómez und Nichols 2013; Poulin 2014). However, these species have long been underrepresented in conservation and ecological studies (Gómez und Nichols 2013; Okamura et al. 2018). Over the past few years, more studies have focused on the role of parasites in ecosystems and revealed that the effects on their hosts are manifold (Gómez und Nichols 2013). Parasites can lead to a decrease in the abundance of hosts, loss of fitness, or behavioral changes in infected individuals (Gómez und Nichols 2013). Overall, parasites are capable of modulating populations and communities of host species and help maintain ecosystem integrity (Gómez et al. 2012). These findings highlight the importance of monitoring parasites in different ecosystems to understand their functions and to consider them in conservation strategies.

Birds are a group of hosts that are of special interest for studies on the spread of diseases and parasitism. Due to their high mobility and the large proportion of migratory species, their parasites and pathogens have the opportunity to spread globally (Fuller et al. 2012). However, information on the spatial distribution of most avian parasites remains limited (Fuller et al. 2012). Mutations in parasites or changes in climatic conditions can lead to changes in distribution ranges, thereby inducing changes in the population of the host species (Atkinson und LaPointe 2009; Garamszegi 2011; Fuller et al. 2012; LaPointe et al. 2012; Loiseau et al. 2013; Alkishe et al. 2017).

Blood parasites of the order Haemosporida are a relatively well-studied group of avian parasites (Valkiūnas 2005; Clark et al. 2014; Ishtiaq 2021). They are found almost globally in most avian families (Valkiūnas 2005; Bensch et al. 2009; Quillfeldt et al. 2011; Clark et al. 2014; Vanstreels et al. 2014; Masello et al. 2018). Haemosporida are known to affect plumage colouration (Romano et al. 2019), body condition (Valkiŭnas et al. 2006), reproductive success (Knowles et al. 2010), and survival (Bennett et al. 1993; Bueno et al. 2010) of avian hosts. The

severity of the infection depends on the period of infection, infection intensity, initial infection dose, and physiological condition of the host (Valkiūnas 2005; Dadam et al. 2019).

In recent years, research on host-parasite interactions has expanded to study single bird species (Ots und Hõrak 1998; Atkinson et al. 2000; Garvin et al. 2003; Dadam et al. 2019; Romano et al. 2019; Names et al. 2021) using a community approach, investigating parasite prevalence differences and the effects of climate change, seasonality, habitat quality, and fragmentation on transmission and infection rates (Bonneaud et al. 2009; Garamszegi 2011; Laurance et al. 2013; Ellis et al. 2015; Ellis et al. 2017; Ferreira Junior et al. 2017; Harvey und Voelker 2019; Ellis et al. 2020; Šujanová et al. 2021). Previous studies on host-parasite interactions at the community level have either been conducted in tropical ecosystems (Ferreira Junior et al. 2017; Fecchio et al. 2018; Lopes et al. 2020) or in wetland-associated ecosystems (Ellis et al. 2020; Šujanová et al. 2021). However, there is a lack of research in temperate managed forests, which limits our understanding of the distribution of host-parasite interactions within the community and how host-parasite interactions vary across different ecosystems. Additionally, human activities, such as logging and urbanization, are known to alter the distribution and abundance of bird species (Schulze et al. 2019) and parasites in ecosystems (Laurance et al. 2013; Santiago-Alarcon et al. 2013). Therefore, further research in temperate-managed forests is necessary to bridge this knowledge gap and provide a more comprehensive understanding of haemosporidian host-parasite interactions at the community level. To our knowledge, host-parasite interactions in European-managed forests have not been previously addressed.

Host-parasite characteristics in bird communities exhibit several features. Some bird families are more frequently affected by parasites. Some show high parasite prevalence or diversity, whereas others host only a few but highly specific parasites, and others are rarely infected (Ellis et al. 2017; Ellis et al. 2020; Šujanová et al. 2021). It has been suggested that abundant host species show a higher parasite diversity due to more closely related species and conspecifics in the same ecosystem, but also a higher parasite prevalence due to higher exposure to vectors (Ellis et al. 2017). However, the connection between parasite prevalence and diversity in bird families is not yet fully understood.

In this study, we focused on the prevalence of haemosporidian parasites and *Trichomonas* spp. in a temperate forest bird community. The latter is reported to infest raptors and pigeons

(Amin et al. 2014) and is caused by microaerophilic protozoa that induce lesions mainly in the upper digestive tract of birds and is transmitted either directly by feeding on conspecific individuals, e.g., in raptors, or indirectly through shared food or water sources (Atkinson et al. 2008; Amin et al. 2014). In 2005, populations of *Chloris chloris* declined in Great Britain due to infection with *Trichomonas gallinae.* Similar infection patterns have been observed in other countries (Peters et al. 2009; Lawson et al. 2012; Amin et al. 2014). *Trichomonas gallinae* is an example of host switching, as it was indirectly transmitted from Columbiformes to Passeriformes, especially to finches, and caused high mortality in their new host species (Lawson et al. 2012). Although infections with *T. gallinae* in passerines are best known from *C. chloris*, other passerines, such as *Fringilla coelebs*, *Erithacus rubecula*, and *Turdus merula*, have also been found to be infected (Quillfeldt et al. 2018).

The aim of this study was to assess and describe the overall prevalence, genetic lineage diversity, and underlying host-parasite network of the endoparasites Haemosporida (*Plasmodium*, *Haemoproteus*, and *Leucocytozoon*) and *T. gallinae* in a European forest bird community during the breeding season. We created a bipartite network and calculated common network parameters to gain insights into interaction diversity and patterns, such as shared lineages within and across avian host families and host specificity within a well-defined community. We hypothesized the following: 1. Parasite prevalence differs between host bird families and is expected to be higher in abundant families such as Paridae and Turdidae. 2. Some families host a higher diversity of parasite lineages, with abundant families exhibiting a higher diversity, and 3. The parasite diversity of bird species affects the parasite prevalence. Here, we also expected that common bird species, such as *Parus major, Erithacus rubecula*, *Sylvia atricapilla*, and *Turdus merula* have high parasite prevalence and lineage diversity. Unveiling the current infection patterns of endoparasites enables the recognition of potential changes within species or communities in the future.

2.3 Material and Methods

The study was conducted in the “Marburg Open Forest,” a 250-ha-sized managed forest in Hesse, Germany. The forest consists of mixed stands dominated by *Fagus sylvatica* and *Quercus robur* mixed with *Pseudotsuga menziesii* and *Picea abies*. Captures were conducted from mid-March to August for four consecutive years, starting in 2019. We set up ten capture sites in the forest and conducted three sampling rounds in 2019 and six sampling rounds were

conducted in each year from 2020 to 2022 (30/60 capture events per year). Birds were captured using a standardized set up with three mist nets (12 m length, 2.5 m height, mesh size 16 mm × 16 mm) in one row per site. Starting in 2020, we used high nets with double the height for every other capture event. At each capture event, the nets were open for three hours during early morning. Birds are not attracted by food or playback. In addition, extra captures were carried out for some species; raptors and crows were caught using the Dho-Gaza method with a mist net (12 m length, 3 m height, mesh size 60 mm × 60 mm) and a stuffed eagle owl placed in front of the net at the forest edges. Additionally, we captured *Phylloscopus sibilatrix* during separate mist-netting events (mist net:12 m length, 2.5 m height, mesh size 16 mm × 16 mm) with playback throughout the forest at locations where the males were singing. All birds were marked with rings to identify the recaptures and permission was obtained from the Heligoland Bird Observatory (Institut für Vogelforschung Heligoland, Germany). We sampled 476 birds belonging to 29 Palearctic species and investigated the prevalence of blood parasites (haemosporidian parasites) and *Trichomonas* spp. using molecular methods.

Sample collection

Blood was sampled from the captured birds with the permission of the regional council of Giessen, Hesse, Germany (V 54–19 c 20 15 h 01 MR 20/15 Nr. G 10/2019) by puncturing the brachial vein, and storing a minimal amount of blood on Whatman FTA Classic cards (Whatman, UK). The blood samples were kept dry until further processing. For the detection of possible infection with *Trichomonas* spp., we took saliva samples with oral swabs which were then stored in *Trichomonas* selective culture medium (*Trichomonas* medium REF EB0861C, OXOID Deutschland GmbH, Wesel, Germany) and incubated at 37°C for five to seven days. Samples were fixed after the first centrifugation (3,200 rpm for 5 min). We added 1 ml of Phosphate-Buffered Saline (PBS), centrifuged once more (3,200 rpm, 5 min), removed the PBS, and stored the samples in a freezer for further processing (Quillfeldt et al. 2018).

Sequencing

Blood and saliva samples were further processed for DNA sequencing in an external laboratory (Ecogenics GmbH, Balgach, CH). DNA was extracted and isolated using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). PCR was conducted using parasite-specific primer

pairs, and blood samples were sequenced using nested PCR (Table 2.1, Hellgren et al. 2004; Quillfeldt et al. 2018). To exclude laboratory errors, positive and negative controls were included in all PCRs. The sequences were uploaded to GenBank® and are available under the accession numbers OQ311003–OQ311317.

Table 2.1: *Primer pairs for the detection of Haemosporida (cyt b) and Trichomonas spp. (ITS1-5.8S-ITS2).*

	Primer	Sequence	Reference
Haemoproteus spp.	HaemNFI	5'-CATATATTAAGAGAAITATGGAG-3'	(Hellgren et al. 2004)
Plasmodium spp. and *Leucocytozoon* spp.	HaemNR3	5'-ATAGAAAGATAAGAAATACCATTC-3'	(Hellgren et al. 2004)
Plasmodium spp. and	HaemF	5'-ATGGTGCTTTCGATATATGCATG-3'	(Hellgren et al. 2004)
Haemoproteus spp.	HaemR2	5'-GCATTATCTGGATGTGATAATGGT-3'	(Hellgren et al. 2004)
Leucocytozoon spp.	HaemFL	5'-ATGGTGTTTTAGATACTTACATT-3'	(Hellgren et al. 2004)
	HaemR2L	5'-CATTATCTGGATGAGATAATGGIGC-3'	(Hellgren et al. 2004)
Trichomonas spp.	TFR1	5'-TGCTTCAGTTCAGCGGGTCTTCC-3'	(Quillfeldt et al. 2018)
	TFR2	5'-CGGTAGGTGAACCTGCCGTTGG-3'	(Quillfeldt et al. 2018)

Data processing and statistics

The resulting sequences (~480 bp) of the mitochondrial cytochrome *b* gene of Haemosporida were trimmed and assembled using the CodonCode Aligner software (CodonCode Corp., Dedham, MA). Except for 53 samples determined only to the genus level because of low-quality regions (possibly indicative of multiple infections), all sequences were free from conflicts in the forward-reverse alignment and had the same length as the reference data of the MalAvi database (Bensch et al. 2009). Samples were assigned to a parasite lineage, using the reference data of the MalAvi database (Bensch et al. 2009) and the 'Identify Sequence' tool of CodonCode Aligner. We compared the sequences to the best match of the reference sequences, and in the case of a 100% match, assigned to a lineage. Some samples (53) could not be matched 100% to one lineage, owing to low-quality regions. The quality of these samples was maintained even after repeated sequencing. Thus, we determined these samples to the genus level and hereafter refer to them as Lineage undetermined. This was the case for

46 *Leucocytozoon* spp. samples and eight *Haemoproteus* spp. samples (one sample was positive for both genera). There were no problems in assigning lineages to any *Plasmodium* spp. positive samples. For repeat samples from recaptured birds, additional lineages were added to the dataset of the respective birds.

To assess and describe our host-parasite community, we assessed the prevalence of each parasite genus in each bird species. We created a stacked bar chart to show the abundance of bird species and the parasite prevalence of the three genera using R (R Core Team 2023) and the package ggplot2 (Wickham 2016). To elucidate interactions between host bird species and parasite lineages, we compiled an interaction network using the bipartite package (Dormann et al. 2008) in R. To describe the host parasite network, we calculated further network-specific parameters and values. The degree indicates the sum of interactions per species or parasite lineage (Dormann et al. 2008), the Paired Differences Index (PDI) provides information on the generality (0) or specificity (1) of the species or parasite lineage (Poisot et al. 2012), and effective partners is the effective number of interacting partners (hosts or parasites) if each partner was equally common (Bersier et al. 2002) at the parasite and host levels (Tables 2.2 and 2.3).

We tested for differences in parasite prevalence (proportion of infected individuals) between bird families by performing a chi-square test in R. To assess differences in lineage diversity (number of haemosporidian lineages) among bird families, we used a chi-square goodness-of-fit test, assuming an even distribution of lineages among all bird families. For both tests, we included only bird families with at least ten individuals, to avoid overestimation of parasite prevalence or lineage diversity based on a small sample size. We calculated a mixed-effects model to test the effect of effective partners on parasite prevalence in bird species using the lme4 package (Bates et al. 2015). We included family as a random effect to correct for closely related species and tested for the impact of family by calculating the marginal and conditional r-squared values.

2.4 Results

Over the course of four breeding seasons, we captured and sampled 483 individual birds (68 recaptures) belonging to 29 species and collected 542 blood and 396 saliva samples. Four of the bird species were represented by only one individual. The species-specific sample sizes are shown in Figure. 2.1.

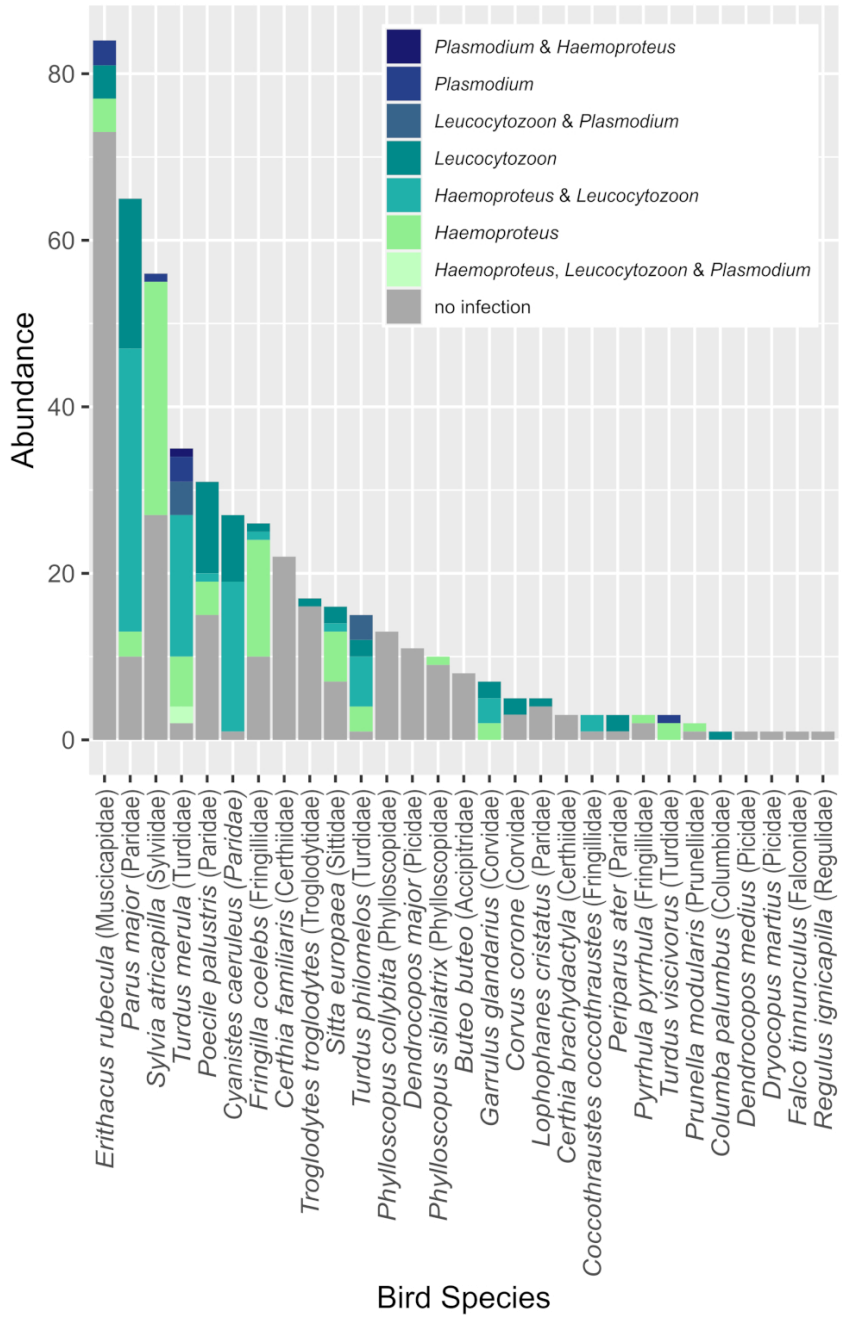

Figure 2.1: Abundance of the bird species (families given) and the corresponding parasite prevalence. The colors indicate haemosporidian genera as indicated in the legend.

We detected Haemosporida infections in almost half of our samples (48.1%, including 53 samples with *genus* undetermined). In 14 bird species, we detected blood parasites in at least half of the individuals (note that one of the 14 species was represented by only one individual; Figure. 2.1). Infections with *Haemoproteus* spp. were the most common (67.7% of infected samples), followed by *Leucocytozoon* spp. infections (59.8%). Only 7.9% of Haemosporida-positive individuals were infected with lineages belonging to *Plasmodium* spp. Distribution of

the three blood parasite genera was unequal within the bird community. *Sylvia atricapilla* was not infected with *Leucocytozoon* spp., whereas all but one (uninfected) of the sampled individuals of *Cyanistes caeruleus* were positive for *Leucocytozoon* spp., most of them in combination with *Haemoproteus* spp. *Plasmodium* spp. were found in only five of the sampled species: three *Turdus* spp., *E. rubecula*, and *S. atricapilla* (Figure. 2.1).

We did not detect any infection with *T. gallinae* within the bird community.

Host-parasite network

Overall, we identified 53 haemosporidian parasite lineages, including 22 lineages of *Haemoproteus* spp., 26 lineages of *Leucocytozoon* spp., and five lineages of *Plasmodium* spp. (Table 2.2). All the detected parasite lineages have been previously described. The most frequent lineage, H_PARUS1 (*H. majoris*, found in 98 birds), was detected in *P. major* (66.3% of all H_PARUS1 positive samples)*, C. caeruleus* (18.4%), *E. rubecula* (1.0%), *Poecile palustris* (5.1%), *Sitta europaea* (7.1%), and *S. atricapilla* (2.0%). The second most common lineages were L_PARUS4 and H_TURDUS2 (22 birds each). L_PARUS4 was found in four host species, namely *C. caeruleus*, *Lophophanes cristatus*, *P. major*, and *P. palustris*, whereas H_TURDUS2 was only found in *T. merula* (Figure 2.2).

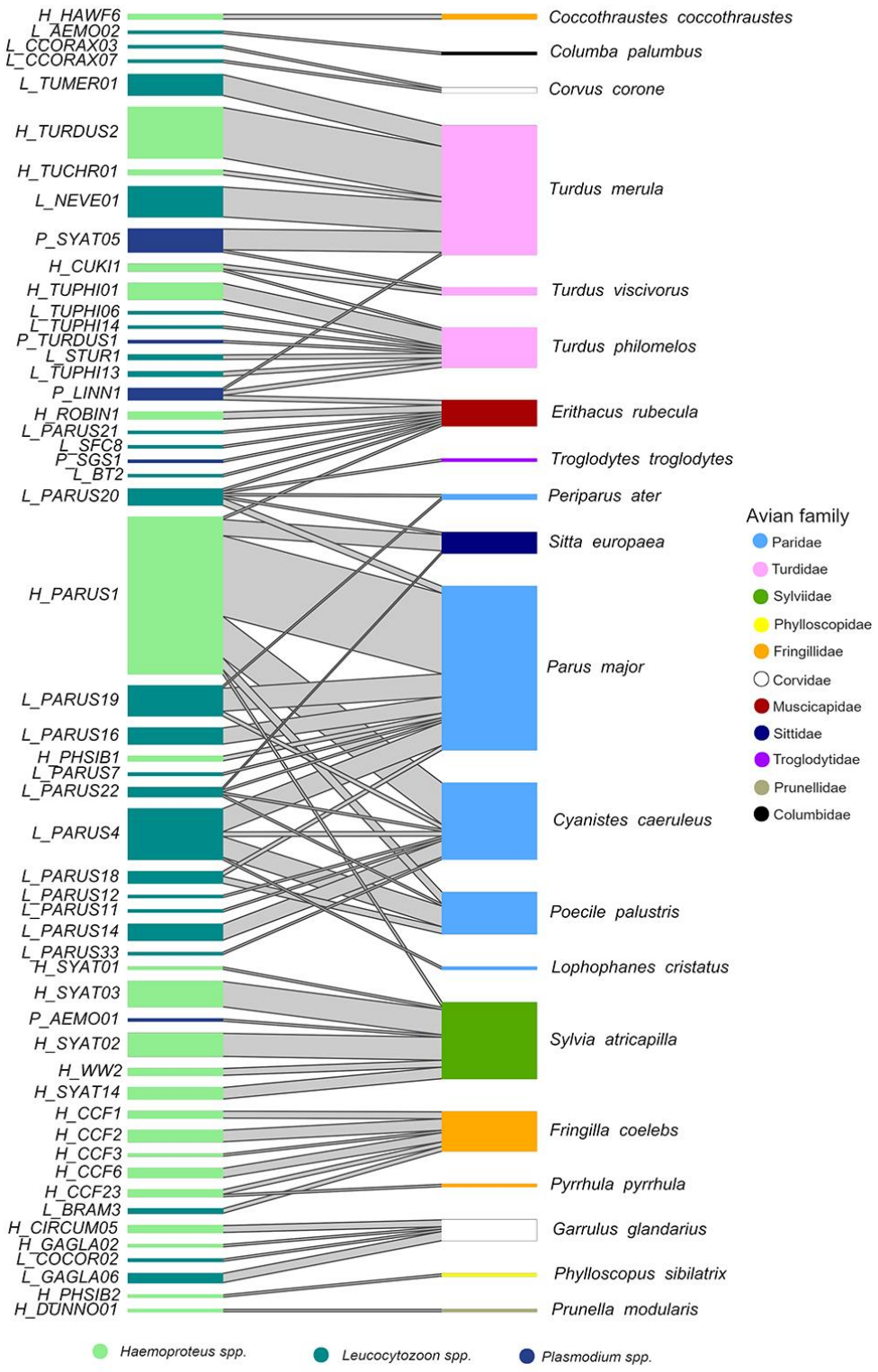

Figure 2.2: Interaction network of parasite lineages (left) and the host bird species (right). The first letter of the lineage indicates the genus: H (Haemoproteus spp.), P (Plasmodium spp.), and L (Leucocytozoon spp.). The width of the boxes indicates the abundance of the species (e.g., C. palumbus = 1, P. major = 68) or lineage (e.g., LAEMO02 = 1, HPARUS1 = 68) and the width of the connecting line indicates the abundance of this interaction (e.g., HPARUS1 – P. major = 35).

At the parasite level the *Haemoproteus* spp. lineage PARUS1 had the highest degree value (6) and compared to the other *Haemoproteus* spp. lineages, it was the most generalist lineage (PDI=0.950) and had the highest number of effective partners (3.616). The most generalized lineage in our network was *Leucocytozoon* spp. PARUS22 (PDI=0.84) whereas PARUS20 was the lineage with the most effective partners (4.37) among the three genera. PARUS20 also had the highest degree (5) of *Leucocytozoon* spp. lineages. In the *Plasmodium* spp. lineages, LINN1 had the most interactions (3), the lowest PDI (0.921), and the most effective partners (2.872).

Table 2.2: Network information on haemosporidian lineages: Degree indicates the sum of hosts per parasite lineage, Paired Differences Index (PDI) provides information on the generality (0) or specificity (1) of this lineage and Effective partners is the effective number of partners if each partner was equally common (Dormann et al. 2008).

Genus	Lineage	Degree	PDI	Effective partners
Haemoproteus	CCF1	1	1	1
	CCF2	1	1	1
	CCF3	1	1	1
	CCF6	1	1	1
	CCF23	2	0.974	1.890
	CIRCUM05	1	1	1
	CUKI1	2	0.974	1.890
	DUNNO01	1	1	1
	GAGLA02	1	1	1
	HAWF6	1	1	1
	PARUS1	6	0.950	3.616
	PHSIB1	1	1	1
	PHSIB2	1	1	1
	ROBIN1	1	1	1
	SYAT01	1	1	1
	SYAT02	1	1	1
	SYAT03	1	1	1
	SYAT14	1	1	1
	TUCHR01	1	1	1
	TUPHI01	1	1	1
	TURDUS2	1	1	1
	WW2	1	1	1
Leucocytozoon	AEMO02	1	1	1
	BRAM3	1	1	1
	BT2	1	1	1
	CCORAX03	1	1	1
	CCORAX07	1	1	1
	COCOR02	1	1	1
	GAGLA06	1	1	1
	NEVE01	1	1	1
	PARUS4	4	0.937	2.952
	PARUS7	1	1	1
	PARUS11	1	1	1

Genus	Lineage	Degree	PDI	Effective partners
	PARUS12	1	1	1
	PARUS14	1	1	1
	PARUS16	1	1	1
	PARUS18	2	0.965	1.960
	PARUS19	3	0.984	1.988
	PARUS20	5	0.930	4.371
	PARUS21	1	1	1
	PARUS22	4	0.842	4
	PARUS33	1	1	1
	SFC8	1	1	1
	STUR1	1	1	1
	TUMER01	1	1	1
	TUPHI06	1	1	1
	TUPHI13	1	1	1
	TUPHI14	1	1	1
Plasmodium	AEMO01	1	1	1
	LINN1	3	0.921	2.872
	SGS1	1	1	1
	SYAT05	2	0.994	1.384
	TURDUS1	1	1	1

On the host side of the network, the species with the highest degree value, and thus most parasite lineages, were *P. major* (9), followed by *E. rubecula* (8), *C. caeruleus* (8), and *T. philomelos* (8). Although we detected a low parasite prevalence in *E. rubecula* (13.1%), it was the most generalized host species (PDI=0.949) and had the highest number of effective partners (7.187).

Table 2.3: Network information on 20 infected bird species: Degree indicates the sum of links per species, Paired Differences Index (PDI) provides information on the generality (0) or specificity (1) of this species and Effective partners is the effective number of partners if each partner was equally common (Dormann et al. 2008).

Species	**Degree**	**PDI**	**Effective partners**
Coccothraustes coccothraustes	1	1	1
Columba palumbus	1	1	1
Corvus corone	2	0.981	2
Cyanistes caeruleus	8	0.984	4.150
Erithacus rubecula	8	0.949	7.187
Fringilla coelebs	6	0.954	5.348
Garrulus glandarius	4	0.976	3.370
Lophophanes cristatus	1	1	1
Parus major	9	0.980	4.875
Periparus ater	2	0.981	2
Phylloscopus sibilatrix	1	1	1
Poecile palustris	4	0.981	3.195
Prunella modularis	1	1	1

Species	Degree	PDI	Effective partners
Pyrrhula pyrrhula	1	1	1
Sitta europaea	3	0.995	1.981
Sylvia atricapilla	7	0.962	5.021
Troglodytes troglodytes	1	1	1
Turdus merula	6	0.970	4.413
Turdus philomelos	8	0.973	5.973
Turdus viscivorus	2	0.990	1.890

Parasite prevalence and lineage diversity

As hypothesized, parasite prevalence was not evenly distributed among bird families (N≥10) in the studied community (chi-square test: χ^2 (10, N=462) = 196.4, p<0.01). The prevalence of haemosporidian parasites was the highest in Turdidae (94.3%, 50/53 individuals) and Paridae (75.6%, 99/131 individuals). In contrast, the Certhiidae (25 individuals) and Picidae (13 individuals) were not infected (Figure. 2.1, Supplementary Material Table 2.4). Likewise, lineage diversity (number of parasite lineages per host family) was significantly different between avian families, as confirmed by the chi-square goodness-of-fit test, χ^2 (10, N=59) = 43.0, p<0.01.

There was no significant effect of effective partners on parasite prevalence (estimate = 0.039; std. error = 0.034, p = 0.257). To estimate the proportion of variation explained by the fixed and random effects, we computed the marginal and conditional R-squared values. While the fixed effect of effective partners had a modest effect (R^2 = 0.03) in explaining the variation in parasite prevalence among infected bird species, the random effect of bird family had a more substantial effect (R^2 = 0.39).

2.5 Discussion

We detected an overall prevalence of haemosporidian parasites of 48% (N = 229/475) in a forest bird community consisting of 29 bird species belonging to 16 avian families and detected 53 haemosporidian lineages. The recorded infections were not equally distributed across the community, with some abundant families and species being more frequently infected (e.g., Turdidae and Paridae) than others. Other rare families, such as Picidae and Certhiidae, were not infected. In contrast to the high prevalence of Haemosporida, we did not detect a single infection of *T. gallinae* in the studied bird community.

Haemosporidian prevalence and host-specificity

Infections with the three haemosporidian genera were commonly found in the forest bird communities. Compared with another study from central Germany, we found a higher prevalence of *Haemoproteus* spp. and *Leucocytozoon* spp. (current study: H: 66.7%, L: 59.8%; Schumm et al., 2019: H: 31.3%, L: 47.5%), whereas *Plasmodium* spp. was less commonly found in our area (current study: 7.9%, Schumm et al. 2019: 12.5%). Another study conducted in a woody wetland in Slovakia found a higher prevalence of *Haemoproteus* spp. and *Plasmodium* spp. (96%) and a lower prevalence of *Leucocytozoon* spp. than those in our study (37%, Šujanová et al. 2021); however, they did not address *Haemoproteus* spp. and *Plasmodium* spp. separately. To date, the drivers of such regional differences are not completely known, but habitat suitability and vector abundance may contribute to these different patterns. For instance, Schumm et al. 2019 discussed that the high prevalence of *Leucocytozoon* spp. could be driven by the number of forests and running water bodies in and around the study area, which are suitable for the reproduction of Simuliidae, the vectors of *Leucocytozoon* spp. (Zahar 1951; Hellgren et al. 2008). However, whether and how climatic and environmental factors determine the distribution of haemosporidian parasites remain unclear. Whereas Ellis et al. 2015) found that the local distributions of *Haemoproteus* spp. and *Plasmodium* spp. were driven by local host populations rather than by environmental factors, Gonzalez-Quevedo et al. 2014) found that the minimum temperature throughout the year was an important predictor of the prevalence of *Plasmodium* spp. Valkiūnas 2005) suggested that the transmission of *Plasmodium* spp. decreased in Europe north of 55° N, potentially because of low temperatures in winter, which is one possible explanation for the low prevalence of *Plasmodium* spp. in our study. The other two Haemosporida genera, *Haemoproteus* spp. and *Leucocytozoon* spp., are known to be transmitted at lower temperatures than those at which *Plasmodium* spp. is transmitted (Mozaffer et al. 2022). The diversity and prevalence of *Leucocytozoon* spp. increased at higher latitudes (Fecchio et al. 2020). The local adaptations of hosts and parasites have also been discussed to cause regional differences (Ellis et al. 2015). To the best of our knowledge, this hypothesis has not been tested yet. It is likely that the regional differences we found were not driven by a single factor, but rather by a combination of climatic factors, vector abundance, and regional host and vector community structures.

There were not only differences in the overall prevalence of Haemosporida infection, but we also found differences in the host-parasite network compared to other studies. When comparing the number of lineages of each haemosporidian genus with those in other studies,

our study revealed a comparably low number of lineages (Ellis et al. 2020; Šujanová et al. 2021). The proportion of lineages per genus, defined as the number of lineages within a genus divided by the total number of lineages found, was found to be very similar between Sweden and Slovakia. Specifically, the proportions of *Plasmodium* spp., *Haemoproteus* spp., and *Leucocytozoon* spp. were 14% / 13%, 46% / 47%, and 40% / 40%, respectively (Ellis et al. 2020; Šujanová et al. 2021). However, our analysis revealed a lower percentage of Plasmodium spp. lineages (9%) and a relatively higher percentage of Leucocytozoon spp. lineages (49%), as reported by Ellis et al. (2020) and Šujanová et al. (2021). This could be caused by fewer host species and a drier and less diverse habitat in our study compared to those of studies from Sweden and Slovakia, where the study area consisted of woody wetlands and forest meadows (Šujanová et al. 2021) or deciduous forests mixed with planted spruce, partly wet soils with small ponds, reed beds, willow thickets, and wet meadows surrounded by dry-grazed grasslands on sandy soils (Ellis et al. 2020).

To further resolve geographical differences in host-parasite interactions, better knowledge of the transmission sites is needed. Migratory birds may have been infected at their stopover sites or their wintering grounds. They have been found to have a higher parasite prevalence and diversity than resident species (Angeli Dutra et al. 2021a; Huang et al. 2022) and have the potential to disperse parasites along their migratory routes, enhancing the spread of parasites (Angeli Dutra et al. 2021b). Previous studies have found that most lineages that infect migratory birds are acquired during migration rather than in their breeding grounds (Huang et al. 2022). However, it is not possible to determine whether the lineages found in this study were transmitted in our forest ecosystem or even in Europe, as some of the host species we sampled were migratory birds. Because of the low proportion of juvenile birds that had not yet migrated and the fact that we did not sample potential vectors, we were not able to disentangle the transmission sites in this study.

In terms of the host specificity of the parasite network presented here, three out of 22 *Haemoproteus* spp. lineages (13.6%), five out of 26 *Leucocytozoon* spp. lineages (19.2%) and two out of five *Plasmodium* spp. lineages (40%) were found in several hosts. This suggests that, in general, *Plasmodium* spp. are generalists, whereas *Haemoproteus* spp. seemed to be more host-specific, which is in line with the findings of other studies (Ellis et al. 2020). However, the most generalist lineage of our network was not a *Haemoproteus* spp. lineage

but a *Leucocytozoon* spp. lineage that was not found in the Swedish study (Ellis et al. 2020) and was slightly more specific in the Slovakian study (Šujanová et al. 2021). Contrary to the hypothesis posited by Poulin (1998) that a higher prevalence of host-specific parasite lineages than generalist lineages, our findings revealed that the most frequently detected lineage (Haemoproteus spp. PARUS1) is a generalist one. Notably, this lineage demonstrated the highest prevalence across three host species, and also infected three additional species, albeit with a lower prevalence. Our findings are in line with those of Hellgren et al. (2009), who reported that parasites exhibiting a broad host range were the most prevalent in their respective host species. The ability to infect multiple host species enhances parasite transmission by increasing the number of potential hosts and the likelihood of successful transmission by vectors (Hellgren et al. 2009). This could explain why some generalist parasites attain a high prevalence within their host species.

Parasite prevalence and lineage diversity of avian hosts

As expected, at the host level of the network, we found significant differences in parasite prevalence and lineage diversity among bird families. Turdidae (94.3%) and Paridae (75.6%) had a particularly high parasite prevalence. Valkiūnas 2005 has earlier described that Passeriformes host the greatest number of haemosporidian lineages. Even within the order Passeriformes, some families such as Fringillidae, Muscicapidae, Paridae, Sylviidae, and Turdidae appear to be more susceptible to haemosporidian parasites (Valkiūnas 2005). All these families, except Muscicapidae, also had a parasite prevalence of over 50% in our study (see Supplementary Material Table 2.4). We found similar patterns at the species level, with some abundant species having particularly high parasite prevalence, such as *C. caeruleus* (96.3%), *P. major* (78.3%), and *T. merula* (94.3%). In contrast, certain Passeriformes, such as Certhiidae, Troglodytidae, and Sittidae, exhibited a comparatively lower parasite prevalence (Valkiūnas 2005). With the exception of the family Sittidae, which had a prevalence of over 50% in our study, our results are consistent with previous findings (Valkiūnas 2005). Several species were not infected at all in our study, such as *P. collybita* or *C. familiaris*. In contrast, Šujanová et al. 2021 detected that two of nine *C. familiaris* individuals were infected with *Plasmodium* spp. in their study in Slovakia.

The high parasite prevalence in certain bird families and species may be driven by several factors. One possible explanation could be that the highly abundant species and families found

in our study are not only common in forests, but are also abundant in different ecosystems (Gedeon et al. 2022). This could lead to an increased vector-host encounter rate (Medeiros et al. 2013; Ellis et al. 2017). Furthermore, the phylogenetic relationships among birds could lead to a high parasite prevalence (O'Connor et al. 2016; Ellis et al. 2020; Huang et al. 2022). Birds with many closely related species have similar immune functions that allow the parasite to utilize several closely related species as hosts, leading to both high parasite prevalence and similar parasite communities (Davies und Pedersen 2008; O'Connor et al. 2016; Clark und Clegg 2017; Ellis et al. 2020). Specifically, phylogenetically closely related host species display comparable haemosporidian parasite prevalence and lineage composition (Ellis et al. 2020; Huang et al. 2022). A common bird family showing this pattern is the Paridae family, which shared a considerable number of lineages in our study. In contrast, only a few shared lineages have been identified in the Turdidae. The reasons for the high degree of lineage sharing among Paridae birds and their rarity in the Turdidae remain to be elucidated. Several possible explanations exist, including a weaker immune response of Paridae species to haemosporidian parasites, making them more vulnerable to haemosporidian infections than Turdidae species (O'Connor et al. 2016). Furthermore, ecological factors could have contributed to this observation, as Paridae birds tend to occur at higher densities, and various species of Paridae coexist within a given ecosystem, whereas Turdidae birds generally have lower densities and do not overlap as much in their use of habitats (Arneberg et al. 1998). The degree to which lineage sharing is influenced by phylogenetic relationships versus ecological and behavioral factors is uncertain. Nevertheless, it is likely that multiple factors contribute to lineage sharing among different families.

As observed in the overall prevalence of haemosporidian genera, there are regional differences in the susceptibility of host species to Haemosporida. These differences could be attributed to several factors. Host-specific factors for high parasite prevalence include breeding abundance, higher exposure to vectors (Kilpatrick et al. 2006; Ellis et al. 2017), and evolutionary distinctiveness, as closely related host species are often infected by the same generalist parasites (Parker et al. 2015; Ellis et al. 2020). In addition to that there are also environmental factors promoting high parasite prevalence and diversity such as habitat quality and seasonal dynamics (Wood et al. 2007; Belo et al. 2011; Ferreira Junior et al. 2017; Šujanová et al. 2021). Parasite prevalence appears to be higher in urbanized areas close to rivers, tropical rainforests, and pastures (Wood et al. 2007; Belo et al. 2011; Ferreira Junior et

al. 2017). To our knowledge, information on parasite prevalence in managed beech forests in Europe is not yet available. This makes it difficult to assess whether the parasite prevalence in our study is high due to habitat type or quality, or whether this is the typical parasite prevalence in managed temperate forests. Furthermore, we only captured and sampled birds during the breeding season, which is known for its high parasite prevalence (Huang et al. 2020) and could also partly explain the high parasite prevalence we found. The condition of birds, resistance to certain parasites, migratory patterns, nest type, and height of the foraging stratum have also been discussed as predictors of parasite prevalence and diversity; however, these were not assessed in this study (Hing et al. 2016; Ellis et al. 2017; Ricklefs et al. 2017; Ilgūnas et al. 2019; Names et al. 2021; Šujanová et al. 2021).

Given that parasite prevalence and lineage diversity differ between bird families and species, we hypothesized that abundant bird species are simultaneously characterized by both high lineage diversity and thus a higher parasite prevalence at the same time (Ellis et al. 2017). In contrast to this hypothesis, we did not find any effect of effective partners on parasite prevalence in the bird species in our network. This theory might apply for some species, such as *P. major* which had both a high parasite prevalence and high lineage diversity. However, *E. rubecula* was our biggest outlier and had a very low parasite prevalence (13.1%) but at the same time a high lineage diversity and was the most frequently caught species in our community. Another study from Hungary reported a similar parasite prevalence in *E. rubecula* (14.9%, Ágh et al. 2019). However, parasite prevalence was not always as low as that in our Hungarian study (Ágh et al. 2019). A study from Sardinia found an almost five-fold higher parasite prevalence in *E. rubecula* which might be due to the lower latitude of the study area and thus different vector communities (61.5%, Pellegrino et al. 2021). Since we did not use microscopy to verify the infection, some of the infections found in *E. rubecula* could have been abortive; thus, the actual lineage diversity might have been lower.

We did not detect an effect of effective partners on parasite prevalence; in fact, the random effect of bird family explained more variance than effective partners. The absence of a correlation between the effective partners and parasite prevalence could be attributed to several factors. There may be a trade-off in parasites between host specificity and the ability to achieve high parasite prevalence (Poulin 1998; Hellgren et al. 2009). Parasite lineages that are highly specific to a single host species may result in high parasite prevalence, whereas

generalist lineages are capable of infecting several hosts but typically do not achieve the same level of prevalence as specific lineages (Poulin 1998). Furthermore, certain host species might be resistant to certain generalist haemosporidian parasites (Ilgūnas et al. 2019), whereas other species are less exposed to vectors, show a low parasite prevalence, and are therefore not well adapted to the parasites, and thus host a larger range of parasite lineages (Ellis et al. 2017). In contrast, generalist parasite lineages were capable of achieving higher levels of prevalence in their host species than in specific lineages (Hellgren et al. 2009). Even though we could not concretely determine why a high parasite prevalence does not predict high lineage diversity, we found that host abundance did not correlate with either of these factors.

The absence of *Trichomonas* spp.

Although we found a high prevalence of haemosporidian parasites, *Trichomonas* spp. was absent in the studied bird community. However, infections with *Trichomonas* spp. in Passeriformes are not very common (Forrester und Foster 2008) despite local outbreaks of trichomonosis in *Chloris chloris* and *F. coelebs* since 2005 (Lawson et al. 2011). Climatic factors have been hypothesized to play a role in the emergence of this disease; however, these were not considered in this study (Amin et al. 2014). Dry weather and low rainfall are suggested to promote the transmission of *Trichomonas* spp., and infections peak in late summer and early fall (Simpson und Molenaar 2006; Bunbury et al. 2007; Lawson et al. 2012). The absence of *Trichomonas* spp. in our study is thus not very surprising, as the bird species of the studied community are not known to be frequent hosts, and we did not take samples in late summer or early spring.

2.6 Conclusions

Our study revealed a high prevalence of haemosporidian parasites in a forest bird community, as well as differences in the susceptibility of different avian families and species, which could potentially be driven by host abundance and habitat choice. The prevalence of different haemosporidian genera, however, differed from those reported in other studies from across Europe. This shows the importance of monitoring host-parasite interactions in different habitats, both natural and human-dominated. The causes of the different susceptibilities of bird families and species remain unclear. As endoparasites are spreading globally in response to climate change and can significantly influence the behavior, reproduction, and mortality of their hosts, our findings highlight the need to monitor host-parasite interactions to better

understand interaction dynamics. The inclusion of vectors and environmental parameters can further help to identify the drivers of regional differences.

2.7 Acknowledgements and declarations

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Author contributions

FS contributed to the conception and design of the study, carried out the field and laboratory work, performed the statistical analyses, and wrote the manuscript. NF, DS, and SR designed and structured the study. PQ, JM, and YS helped with conceptual and analytical approaches and supported the lab and fieldwork. MB, KL, DS, and SR contributed to fieldwork. YS introduced and helped with both field and laboratory work. NF, DS, and SR contributed to the statistical analysis. All authors contributed to manuscript revision and have read and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary materials. The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2023.1107736/full#supplementary-material. The DNA sequences were uploaded to GenBank® and are available under the accession numbers OQ311003–OQ311317 and can further be found at the MalAvi Database (Bensch et al. 2009).

Ethics statement

The animal study was reviewed and approved with permissions for animal testing by regional council Giessen, Hesse, Germany (V 54-19 c 20 15 h 01 MR 20/15 Nr. G 10/2019).

Chapter III: Identifying and Counting Avian Blood Cells in Whole Slide Images via Deep Learning

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3.1 Simple Summary

Avian blood analysis is crucial for understanding the health of birds. Currently, avian blood cells are often counted manually in microscopic images, which is time-consuming, expensive, and prone to errors. In this article, we present a novel deep learning approach to automate the quantification of different types of avian red and white blood cells in whole slide images of avian blood smears. Our approach supports ornithologists in terms of haematological data acquisition, accelerates avian blood analysis, and achieves high accuracy in counting different types of avian blood cells.

3.2 Abstract

Avian blood analysis is a fundamental method for investigating a wide range of topics concerning individual birds and populations of birds. Determining precise blood cell counts helps researchers gain insights into the health condition of birds. For example, the ratio of heterophils to lymphocytes (H/L ratio) is a well-established index for comparing relative stress load. However, such measurements are currently often obtained manually by human experts. In this article, we present a novel approach to automatically quantify avian red and white blood cells in whole slide images. Our approach is based on two deep neural network models. The first model determines image regions that are suitable for counting blood cells, and the second model is an instance segmentation model that detects the cells in the determined image regions. The region selection model achieves up to 97.3% in terms of F1 score (i.e., the harmonic mean of precision and recall), and the instance segmentation model achieves up to 90.7% in terms of mean average precision. Our approach helps ornithologists acquire haematological data from avian blood smears more precisely and efficiently.

3.3 Introduction

Automated visual and acoustic monitoring methods for birds can provide information about the presence and the number of bird species (Höchst et al. 2022) or individuals (Ferreira et al. 2020) in certain areas, but analysing the physiological conditions of individual birds allows us to understand potential causes of negative population trends. For example, measuring the physiological stress of birds can serve as a valuable early warning indicator for conservation efforts. The physiological conditions and the stress of birds can be determined in several ways, e.g., by assessing the body weight or the fat and muscle scores in migratory birds (Johnson et al. 1985; van Balen 2002). Other frequently used methods are investigating the parasite loads, measuring the heart rates, and measuring the levels of circulating stress hormones, such as corticosterone (Thompson et al. 1997; Froget et al. 2001; Schoenle et al. 2017; Sorenson et al. 2017; Rösner et al. 2023). Depending on the research questions studied, these methods can be a good choice for assessing long-term stress or the investment in immunity.

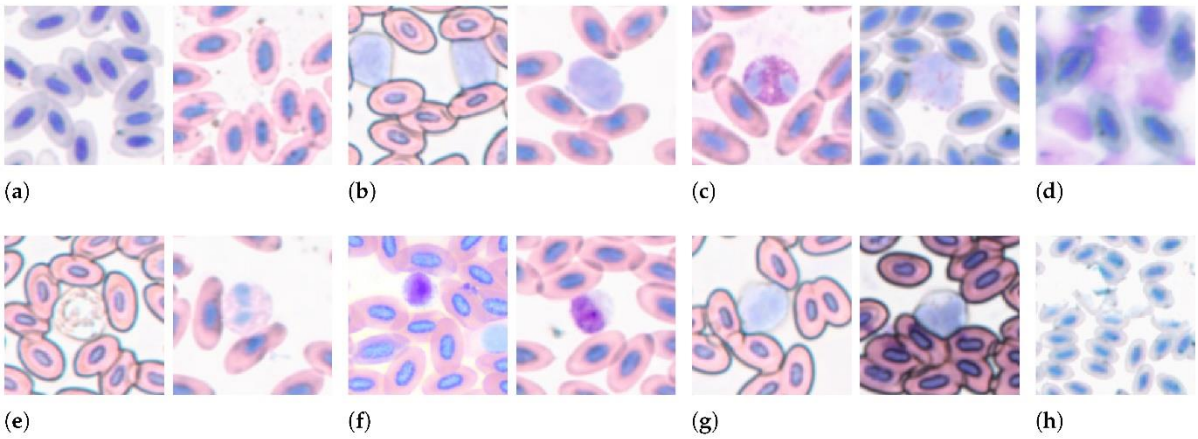

Figure 3.1: Examples of different cell types: (a) erythrocytes of a Blue Tit (*Cyanistes caeruleus*) and a Eurasian Blackcap (*Sylvia atricapilla*); (b) lymphocytes of a Common Blackbird (*Turdus merula*) and a Common Buzzard (*Buteo buteo*); (c) eosinophils of a Common Buzzard and a European Robin (*Erithacus rubecula*); (d) stain remnants and lysed cells in the blood sample of a Black Woodpecker (*Dryocopus martius*); (e) heterophils of a Common Blackbird and a Common Buzzard; (f) basophils of a Common Blackbird and a Common Buzzard; (g) monocytes of a Common Blackbird and a European Robin and (h) ruptured cells in a blood sample of a Eurasian Blue Tit. The blood smears were stained with Giemsa and scanned at 40× magnification.

The method investigated in this article comprises analysing blood smears and counting blood cells (Davis et al. 2008). Not only are white blood cells, i.e., leukocytes, an important part of

the immune system of vertebrates such as mammals or birds, but also the composition of leukocytes is known to change in response to elevated stress hormones (glucocorticoids) and can, therefore, be used to assess stress levels (Davis et al. 2008). In particular, the ratio of heterophils to lymphocytes (H/L ratio) is considered to be a well-established stress index for assessing long-term stress in birds (Davis et al. 2008; Skwarska 2019). Since the H/L ratio changes only 30 to 60 min after the onset of an acute stress event, it is possible to measure stress without mirroring the influence of the capture event (Cīrule et al. 2012). It is also possible to calculate the leukocyte concentration (leukocytes per 10,000 erythrocytes) or the concentration of specific leukocyte cell types for gaining an understanding of the current health status of a bird and the investment in immunity (Salvante 2006; Masello et al. 2009; Wojczulanis-Jakubas et al. 2012).

Leukocyte counts are quite cost-effective since they do not require complex laboratory techniques. However, evaluation under the microscope often requires manual interpretation by human experts, is time-consuming, and can only assess small portions of the entire smear. Typically, leukocytes are counted until 100 leukocytes are reached (Ruiz et al. 2002). Consequently, the counted values and subsequent ratios are not always reproducible, and the result depends on the section counted. Furthermore, the method is prone to observer errors. Therefore, there is an urgent need for automated methods to perform leukocyte counts in avian blood smears.

Bird and human blood cell analysis have some aspects in common. The counted leukocytes are similar: lymphocytes, eosinophils, basophils, and monocytes can be found in mammalian as well as avian blood. However, there are some significant differences that make the automated counting of avian blood cells more difficult (Hawkey et al. 1989). The neutrophils in human blood are equivalent to heterophils in birds. One of the main differences between bird and human blood, however, is the presence of nuclei in bird erythrocytes (i.e., red blood cells) and thrombocytes, whereas there is no nucleus in mammalian erythrocytes and thrombocytes (Hawkey et al. 1989). The presence of a nucleus in erythrocytes makes the cell identification process more complicated since lysed and ruptured erythrocytes can be mistaken for other cell types. Lastly, during ornithological field studies, the bird blood samples are usually not taken in a sterile environment, leading to dirt contaminating the smears. Such contaminants and stain remnants can further lead to confusion. Because of these differences

from human blood and the associated challenges, it is necessary to develop dedicated solutions instead of relying on existing machine learning approaches for human blood analysis to automatically analyse bird blood samples.

A solid understanding of the different leukocyte types is necessary when analysing avian blood samples since some are quite similar to each other. Figure 1 shows examples of each blood cell type as well as two challenging anomalies, i.e., stain remnants and lysed cells (Figure 1d) as well as ruptured cells (Figure 1h). Lymphocytes appear small, round, and blue in blood smears and are most common in passerine birds. Their nuclei usually take up more than 90% of the cell (see Figure 1b). Heterophils can be identified by their lobed cell nuclei and rod-shaped granules in the cytoplasm, as shown in Figure 1e. In birds, heterophils and lymphocytes make up approximately 80% of the leukocytes (Rupley 1997). Eosinophils are similar to heterophils but have round granules (see Figure 1c). Basophils can be recognized by their purple-staining granules, as shown in Figure 1f, but they are rare to find. Monocytes are larger cells that can be confused with lymphocytes, but their nucleus often has a kidney-shaped appearance and takes up only up to 75% of the cell (see Figure 1g; Davis 2012). Additionally, it is important to be aware of possible variations regarding the morphology and staining characteristics of these cell types between different avian species, which may affect their identification and interpretation.

Avian blood counts are still mostly obtained manually. However, there are several approaches for more systematic, automated ways of counting avian blood cells. For instance, Meechart et al. (2019)) developed a simple computer vision algorithm based on Otsu's thresholding method (Otsu 1979) to automatically segment and count erythrocytes in chicken blood samples. Beaufrère et al. (2013) used image cytometry, i.e., the analysis of blood in microscopy images, in combination with the open-source software CellProfiler (Jones et al. 2009; Kamentsky et al. 2011) to classify each cell using handcrafted features as well as machine learning algorithms. However, they stated their results were not satisfactory.

Another way of automating avian blood counts is the use of hardware devices for blood analysis. For example, the Abbott Cell-Dyn 3500 haematology analyser (Abbott, Abbott Park, IL, USA) was used in studies analysing chicken blood samples (Post et al. 2003; Lilliehöök et al. 2004). The Cell-Dyn 3500 works on whole blood samples and relies on flow cytometry, i.e., the

analysis of a stream of cells by a laser stream and electric impedance measurements. The device was standardized for poultry blood.

The CellaVision® DC-1 analyser (CellaVision AB, Lund, Sweden) scans blood smears and pre-classifies erythrocytes as well as leukocytes. In combination with the proprietary CellaVision® VET software (CellaVision, AB, Lund, Sweden), the device can be used to analyse animal blood, including bird blood. However, the pre-classification results still need to be verified by a human expert. The device has a limited capacity of a single slide and is able to process roughly 10 slides per hour, according to the manufacturer (CellaVision AB, Lund, Sweden). This throughput does not appear to reduce turnaround times in (human) blood analysis (Lee et al. 2023). Yet, in a distributed laboratory network, the device could indeed contribute to reduced turnaround times (Mayes et al. 2023).

In the last decade, deep learning models, in particular convolutional neural networks (CNNs), have become the state of the art in many computer vision tasks, such as image classification, object detection, and semantic segmentation. These deep neural networks are highly suitable for image processing since they can learn complex image features directly from the image data in an end-to-end manner. Apart from their success in natural image processing, they have also contributed to biological and medical imaging tasks, e.g., in cell detection and segmentation (Ronneberger et al. 2015; Korfhage et al. 2020), blood sample diagnostics (Chola et al. 2022; Kittichai et al. 2023), histopathological sample diagnostics (Guo et al. 2023), such as breast cancer detection (Rashmi et al. 2021), and magnetic resonance imaging (MRI) analysis (Chattopadhyay und Maitra 2022).

However, only a few deep learning approaches are available for avian blood cell analysis. For instance, Govind et al. 2018 presented a system for automatically detecting and classifying avian erythrocytes in whole slide images. Initially, they extract optimal areas from the whole slide images for analysing erythrocytes. In the first step, regions are chosen from low-resolution windows using a quadratic determinant analysis classifier. These optimal areas are then refined at higher resolution using an algorithm based on binary object sizes. This algorithm identifies overlapping cells that need to be split. The actual separation is conducted in a multi-step handcrafted algorithm. Intensity- and texture-based features are used to distinguish between erythrocytes and leukocytes, but the latter are not actually detected. In the final step, all detected erythrocytes, i.e., solitary and separated from clumps, are classified.

This is the only part of the approach that relies on deep learning. Each detected cell is cropped and fed to a GoogLeNet deep neural network (Szegedy et al. 2015). The resulting model can classify the detected erythrocytes as mammalian, reptilian, or avian. Furthermore, the model can categorize erythrocytes into one of thirteen species. However, only one of these is a bird species.

Kittichai et al. 2021 used different CNN models to detect infections of an avian malaria parasite (*Plasmodium gallinaceum*) in domestic chickens. Initially, a YOLOv3 (Redmon und Farhadi 2018) deep learning model was used to detect erythrocytes in thin blood smear images. Then, four CNN architectures were employed for the classification of the detected cells to characterize the different avian malaria blood stages.

However, to the best of our knowledge, there is no hardware-independent and publicly available approach for the automated segmentation and classification of avian blood cells, i.e., erythrocytes as well as leukocytes.

In this article, we present a novel deep learning approach for the automated analysis of avian blood smears. It is based on two deep neural networks to automatically quantify avian red and white blood cells in whole slide images, i.e., digital images produced by scanning microscopic glass slides (Parwani 2022). The first neural network model determines image regions that are suitable for counting blood cells. The second neural network model performs instance segmentation to detect blood cells in the determined image regions. For both models, we investigate different neural network architectures and different backbone networks for feature extraction in cell instance segmentation. We provide an open-source software tool to automate and speed up blood cell counts in avian blood smears. We make the annotated dataset used in our work publicly available, along with the trained neural network models and source code (umr-ds). In this way, we enable ornithologists and other interested researchers to build on our work.

3.4 Material and Methods

We present a deep learning approach for automatically identifying and counting avian blood cells. The approach is divided into three main phases. Figure 2 gives an overview of the entire process from acquiring blood smears to automatically emitting a blood cell count for a whole slide image. In the first phase, avian blood samples are acquired and digitized. Next, the

resulting images are split into tiles that are uploaded to a web-based annotation tool used by a human expert to thoroughly annotate tiles for both tasks, i.e., tile selection and cell instance segmentation. In the second phase, adequate deep neural networks are trained for both tasks. The deep neural network models assist a human expert during annotation by providing pre-annotations for further labelling. In the third phase, the trained deep neural network models are applied to process whole slide images that were not used during training. These images are split into tiles that are analysed by the tile selection model. Only tiles approved as countable are forwarded to the instance segmentation model. The final blood cell counts are determined based on the outputs of the instance segmentation model.

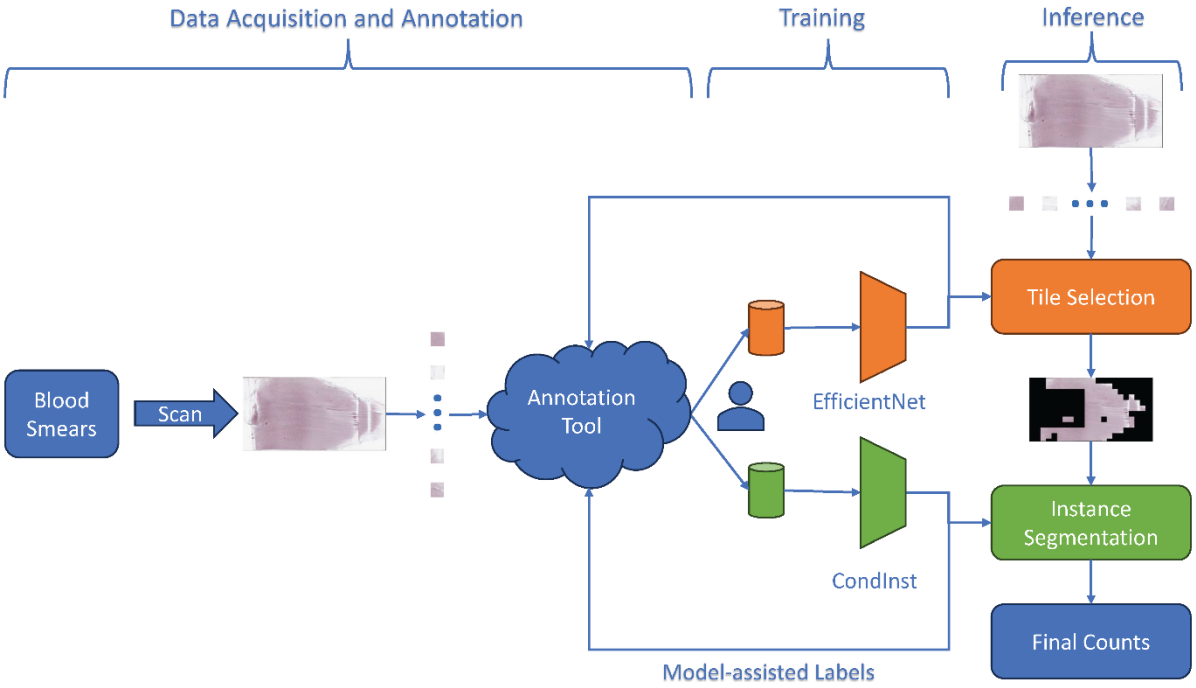

Figure 3.2: Overview of our approach for counting avian blood cells in whole slide images. Data Acquisition: Blood smears are prepared and scanned. The images are then cut into tiles and annotated by a human expert via a web-based annotation tool. Training: The neural network models are trained using annotated data for tile selection and instance segmentation. The models also assist in iteratively annotating images. Inference: An input image is tiled and fed into the tile selection model. Countable tiles are passed to the instance segmentation model before the final counts are determined.

Data Acquisition and Annotation

To be able to train deep learning models at scale, we used avian blood smear samples from an ornithological field study in the Marburg Open Forest (MOF), a 250-hectare beech-dominated forest in Central Hesse, Germany. The data collection took place over four

consecutive years, from 2019 to 2022, during the breeding seasons of the entire forest bird community (29 species of 16 families and 5 orders) between mid-March and August. The birds were captured using mist nets 12 m in length and 2.5 m in height; the mesh size was 16 × 16 mm. Each bird was marked with a ring for re-capture identification with the necessary permissions obtained from the Heligoland Bird Observatory (Institut für Vogelforschung Heligoland, Germany). A blood sample was taken within 30 min from capture by puncturing the brachial vein and using heparinized capillary tubes, following the animal testing approval granted by the regional council of Giessen, Hesse, Germany (V 54–19 c 20 15 h 01 MR 20/15 Nr. G 10/2019). The blood was then used to create one or two whole blood air dry smears of each bird right after sampling in the field. In the laboratory, the blood smears were fixed in methanol within 24 h and stained with Giemsa within 21 days, following standard protocols (Robertson und Maxwell 1990).

Our work relies on two data sources. We created a first, small dataset consisting of 160 images through manual acquisition. The images were digitized with a Zeiss AxioCam ERc 5s Rev.2 (Carl Zeiss AG, Oberkochen, Germany) in combination with a Zeiss Primo Star microscope (Carl Zeiss AG, Oberkochen, Germany) at 100× magnification with oil immersion and saved in the PNG image format with a resolution of 2560 × 1920 px. However, this method is not suitable for creating a large dataset since there is a significant manual effort involved, which makes the method very time-consuming.

To create a second, larger dataset, we digitized one or two blood smears per bird during the initial capture and, in recapture cases, we digitized another one or two blood smears of the same bird by scanning the blood smears with a Leica Aperio AT2 Scanner (Leica Biosystems Nussloch GmbH, Nussloch, Germany) at 40× magnification. We selected the highest quality smear of each bird per capture for our analysis. Aperio scanners generate high-resolution images that are stored in the SVS file format, which consists of a series of TIFF images. The first image is always the full-resolution scan. In subsequent layers, it is split into tiles that are decreasing in size with each layer. Overall, we obtained 527 whole slide images from 459 individual birds of 29 species. These images range from 47,807 px to 205,176 px in width and from 35,045 px to 93,827 px in height. To be able to process these huge images with deep learning models, we used OpenSlide (Goode et al. 2013) to crop them into tiles.

The complete and accurate annotation of the dataset is crucial for training a high-quality deep learning model. To reduce the impact of human errors and eliminate consistency issues in the annotations, we relied on a single human expert for the labelling task. In the following, we describe the annotation process in more detail for both of our tasks, i.e., tile selection and cell detection and segmentation.

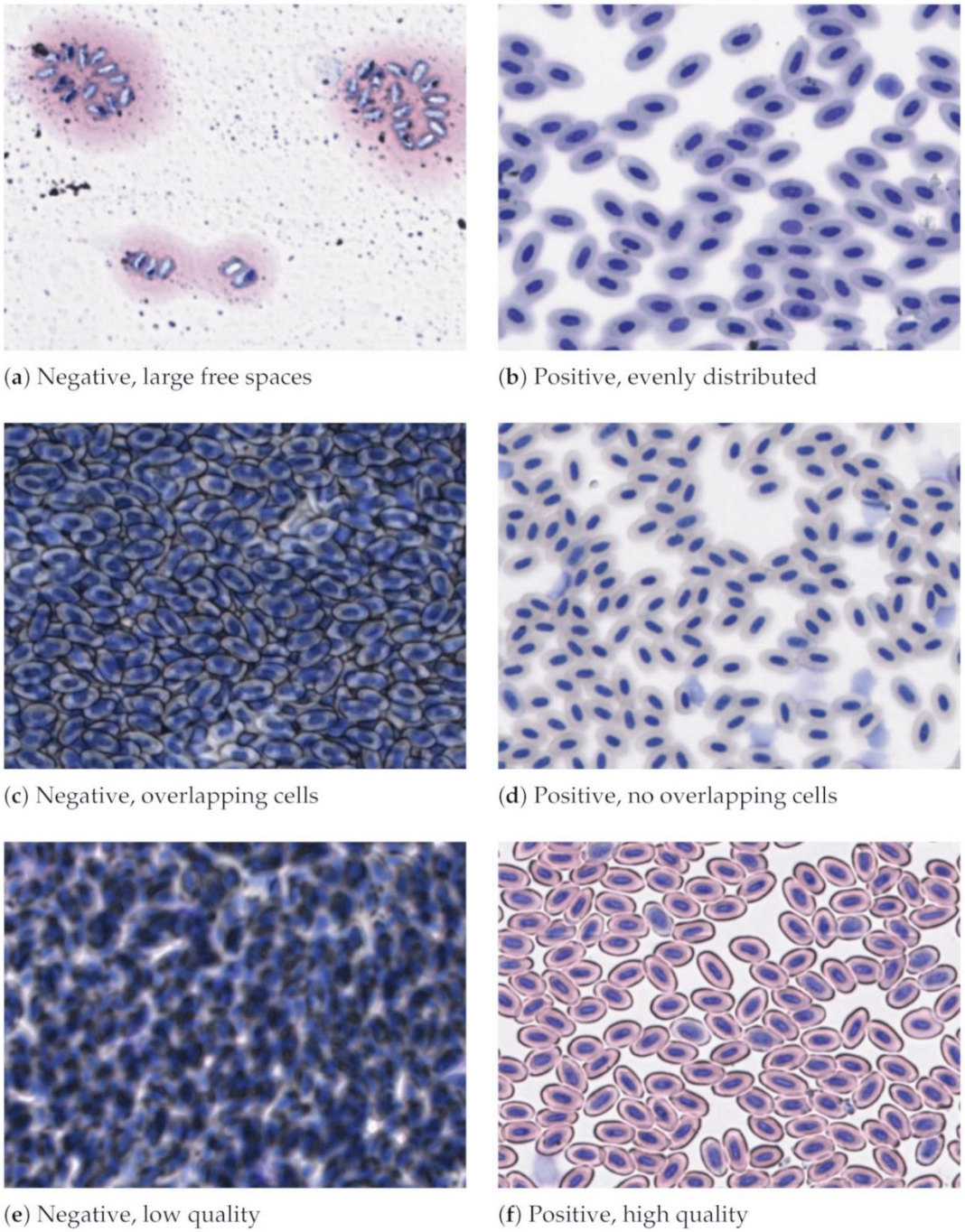

Figure 3.3: Positive and negative examples for image tiles that are suitable for counting cells. Subfigures (a), (c), and, (e) show non-countable examples, while Subfigures (b), (d), and, (f) show countable tiles.

There are several criteria for classifying an avian blood smear image tile as either countable or non-countable. Figure 3 shows examples of positive (i.e., countable) and negative (i.e., non-countable) image crops. Cells should be equally distributed, as shown in Figure 3b, without large empty spaces, as shown in Figure 3a. Furthermore, there should be only a few overlapping cells and especially no overlapping nuclei, as contrasted in Figure 3c,d. In general, good image quality is desirable (Figure 3f,e). To be able to train a deep learning model to classify blood smear image tiles as countable or non-countable, a human expert manually selected image tiles and classified them as countable or non-countable. This process led to a dataset consisting of 2288 positive and 2372 negative examples. While it is sufficient for the tile selection task to simply annotate each sample to be countable or not, we fully annotated the dataset for detection with segmentation masks instead of using bounding boxes. Hence, each single cell instance needs to be precisely covered by a mask and tagged with the corresponding class label. Although this annotation method takes even more time, it improves the performance of the final model (He et al. 2017). Providing exact cell boundaries is particularly beneficial in crowded image regions, where several bounding boxes may overlap.

We used the web platform Labelbox (Labelbox 2023) for annotating images of our datasets. The instance segmentation dataset was annotated in an iterative, model-assisted manner. This means that we used the tile selection network to propose regions to be annotated and eventually selected them based on how many rare cells had been detected by an intermediate instance segmentation model. In the very first iteration, we used a superpixel algorithm to generate simple instance masks. In each iteration, we uploaded the corresponding instance segmentation masks to Labelbox to be refined by our human expert. This procedure significantly reduces the time needed to fully annotate an image file with masks and class labels compared to annotating from scratch. Overall, we went through four iterations of labelling. For the annotated cell instances, we established two primary categories: erythrocyte, with only the nucleus annotated, and leukocyte. The latter was further split into five subtypes, namely, lymphocyte, eosinophil, heterophil, basophil, and monocyte. Thrombocytes were not explicitly annotated; they were considered to be part of the background during training. Thus, our trained neural network model can distinguish between non-relevant thrombocytes and other annotated cell types, e.g., erythrocytes. By annotating only the nucleus of each erythrocyte rather than the entire cell including the cytoplasm, we maintained the option to label parasite-infected instances individually in future work. Cells

infected with parasites may be annotated by masking the entire cell including the cytoplasm. One erythrocyte can be simultaneously counted as both an erythrocyte and a cell with blood parasite because of the distinct annotation regions.

Table 3.1: Summary of annotated bird blood cells across 24 bird species and 1,810 blood smear images. Numbers are given for each order of birds occurring in the dataset, and for each blood cell type, i.e., erythrocytes as well as five subtypes of leukocytes.

Order	Erythrocyte	Lymphocyte	Eosinophil	Heterophil	Basophil	Monocyte	Overall
Accipitriformes	26,867	254	504	208	28	5	27,866
Columbiformes	329	1	1	5	0	0	336
Falconiformes	245	2	0	0	0	0	247
Passeriformes	192,188	1,638	611	815	130	76	195,458
Piciformes	2,573	15	9	12	0	0	2,609
Σ	222,202	1,910	1,125	1,040	158	51	226,781

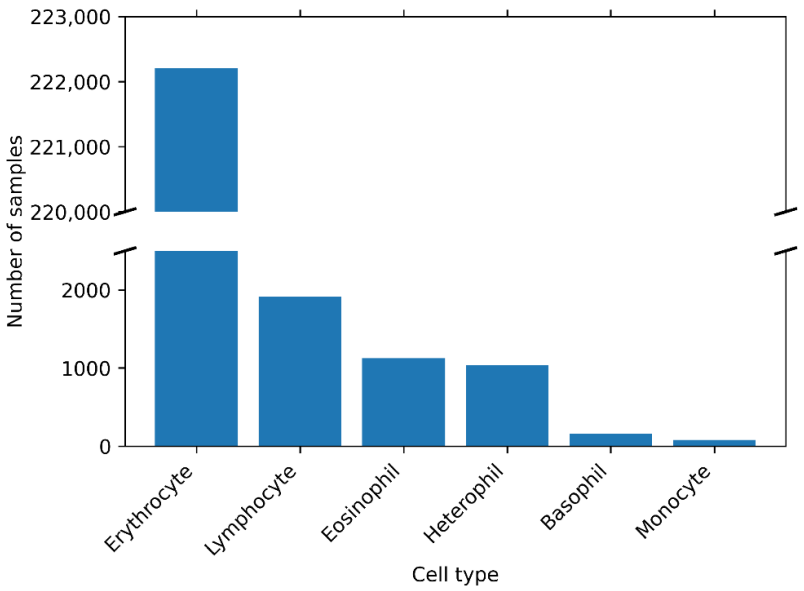

Figure 3.4: Distribution of annotated bird blood cell types. The plot shows how the annotated cell instances are spread among different cell types, i.e., erythrocytes, lymphocytes, eosinophils, heterophils, basophils, and monocytes.

Overall, our segmentation dataset consisted of 1810 fully annotated images. As Table 3.1 shows, the dataset contained 226,781 annotated cell instances that were unevenly spread across 5 taxonomic bird orders, namely, Accipitriformes, Columbiformes, Falconiformes, Passeriformes, and Piciformes. The orders Passeriformes and Accipitriformes dominated the

dataset. Moreover, with a share of 98% of all cells, erythrocytes were by far the most frequent type of blood cells in our dataset. Among the leukocytes, the subtypes were also not distributed equally. While lymphocytes, eosinophils, and heterophils numbers were between 1000 and 2000 samples, basophils and monocytes were expectedly very rare, as Figure 4 demonstrates. This imbalance is often challenging for machine learning approaches. The annotation of the instance segmentation dataset took our human expert more than 70 h.

Deep Learning Approach

Our novel approach for analysing whole slide avian blood smear images consists of two stages, i.e., tile selection and instance segmentation. The tile selection process is shown in Figure 5. Initially, we decompose the input whole slide image into tiles. For each tile, we perform a binary classification to ensure that the contained cells fulfil the requirements to be countable. Next, an instance segmentation model is applied to all tiles that are classified as countable. This model detects all cells in the image and classifies each one as either an erythrocyte or as one of the subtypes of leukocytes. Figure 6 illustrates this procedure. Each step is explained in more detail in the following sections.

Tile Selection

For the tile selection model, we used EfficientNet (Tan und Le 2019) as our architecture. EfficientNet is a family of neural network architectures that has proven to be an excellent choice for image classification. We experimented with the two smallest versions of EfficientNet, namely EfficientNet-B0 and EfficientNet-B1. In a pre-processing step, we randomly applied data augmentation to prevent our model from overfitting the training data. Besides random contrast, random hue, random cropping, and horizontal as well as vertical flipping, these augmentations included elastic transformations that have proven to be very beneficial for cell recognition tasks (Ronneberger et al. 2015; Korfhage et al. 2020). The input size of our model was 512 × 384 px. Our training was based on a well-established pre-trained ImageNet model and fine-tuned in two phases using the Adam optimizer (Kingma und Ba 2014) and binary cross-entropy loss. By experimenting with different learning rates, we found an initial learning rate of 1×10^{-4} to work best in our case. During the first training phase, we kept the majority of the model parameters fixed. We made an exception for the last layer, where we introduced a new set of weights. This approach ensured that the new layer would

not interfere with the pre-trained weights in the rest of the model. In this way, the randomly initialized last layer could adapt to the training data. We trained the network for 20 epochs, i.e., iterating with the whole training dataset 20 times. In the second training phase, we lowered the initial learning rate by a factor of 10 and trained the last 20 layers of the model to ensure that the model would learn useful features for the tile selection task. The loss converged again after up to 30 more epochs. Furthermore, in both phases, the learning rate was reduced whenever the validation accuracy stagnated in order to help the model find the optimal set of weights.

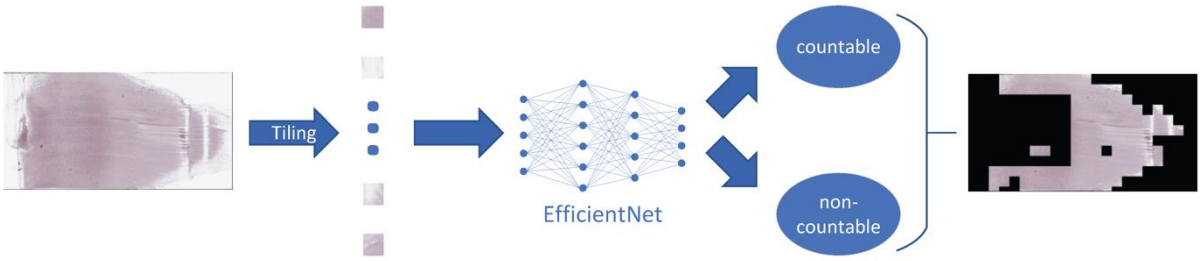

Figure 3.5: Overview of the tile selection phase. The original image is split up into single tiles. Each tile is fed into the EfficientNet CNN model that classifies it as either countable or non-countable. We finally visualize the results by blacking out all tiles classified as non-countable. For visualization purposes, we aggregated 16 × 16 tiles to one patch in the depicted output.

Detection and Segmentation

For the instance segmentation task, we used the CondInst architecture (Tian et al. 2020), as shown in Figure 6. This neural network is based on the anchor-free object detector FCOS (Tian et al. 2022) that tackles object detection in a fully convolutional way. To solve object detection or instance segmentation tasks, many approaches rely on anchor boxes and proposals, e.g., Faster R-CNN (Ren et al. 2015) and most versions of YOLO (Redmon und Farhadi 2017).

For instance, Faster R-CNN generates bounding box proposals based on pre-defined anchor boxes. Anchor boxes of different scales and aspect ratios are placed in each area of the image and are assigned a score based on how likely they are to contain a relevant object. High-scored proposals are resized to a common size and processed in parallel in two different branches of the network, the so-called heads. One refines the proposed bounding boxes, i.e., the box regression head, while the other predicts the corresponding class label, i.e., the classification head.

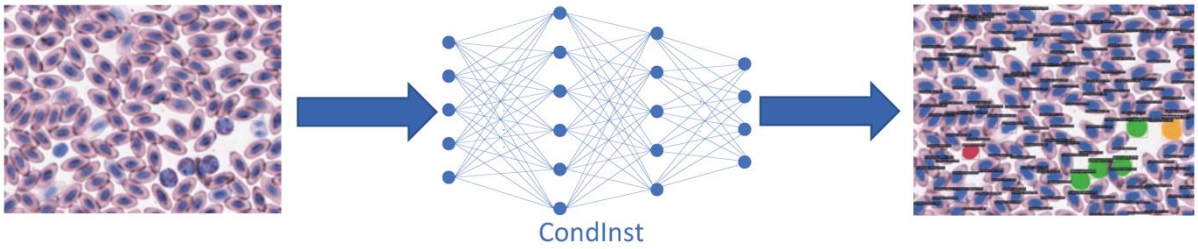

Figure 3.6: Overview of the cell instance segmentation phase. Countable image tiles serve as inputs to the CondInst instance segmentation model. On the right, we visualize the predictions made by the model. Each colour corresponds to one cell type. In the example, the model recognized one lymphocyte (red), one heterophil (orange), four eosinophils (green), and many erythrocytes (blue) reliably.

However, using anchor boxes has several drawbacks. First, deep neural networks based on anchor boxes are sensitive to the choice of hyperparameters. For example, since anchor box scales and aspect ratios are fixed, they cannot easily adapt to new tasks. Furthermore, such networks are computationally inefficient. They produce many negative boxes and have to calculate many Intersection over Union (IoU) values to find the optimal proposals. The object detector FCOS (Tian et al. 2022) relies on neither anchor boxes nor proposals. Instead, one of its heads simply predicts a 4D vector regressing the bounding box centred at this location.

While fully convolutional networks have been commonly used for semantic segmentation for many years, the CondInst architecture (Tian et al. 2020) successfully applies this type of neural network to instance segmentation. To be able to predict instance segmentation masks rather than bounding boxes, CondInst adds a mask branch to FCOS and inserts a so-called controller head, along with the classification and bounding box heads for each location (x, y). The controller head dynamically generates convolutional filters specifically built for each instance in an image by predicting its parameters. In this way, the mask branch becomes instance aware, i.e., it can predict one segmentation mask for each object instance in the image. This strategy yields very good results for irregular shapes that are challenging to tightly enclose within a bounding box.

To train our model, we used two different kinds of input data. The first part of our training dataset consisted of 138 images with a resolution of 2560 × 1920 px from the manually acquired data source. Since these images were captured with a magnitude different from the one used for the whole slide images, we needed to choose the crop tile size such that they

contained a comparable number of similarly sized cells. This resulted in a tile size of 512 × 384 px.

In a pre-processing step, we resized each image to 1066 × 800 px, matching the maximal short-edge input size of CondInst. Furthermore, we applied extensive data augmentation to enrich the dataset and prevent the model from overfitting to the training data. In particular, we applied random horizontal and vertical flipping as well as random adaptations of brightness, contrast, and saturation in an empirically chosen range of 0.7 to 1.3, respectively. Additionally, we applied random elastic transformations that are explicitly useful for cell segmentation tasks (Ronneberger et al. 2015; Korfhage et al. 2020) since they produce realistic alternations of the cells by using an $x \times x$ grid overlay and distorting it with random displacement vectors. We empirically chose $x \in \{6,7,8,9\}$. The order of magnitude ranged from 5 to 10. Through visual inspection, we made sure that deformations based on these parameters did not produce unrealistic cell structures.

We built our CondInst model based on a ResNet-101 backbone architecture. To enable transfer learning, the entire CondInst network was initialized with weights pre-trained on the COCO (Common Objects in Context) object detection dataset (Lin et al. 2014). Hence, we lowered the initial learning rate by a factor of 10, which resulted in a learning rate of 0.001. To match the number of classes, we modified the classification head accordingly. We optimized the network for 16,600 iterations at a batch size of 4, i.e., more than 50 epochs. When approaching the end of the training, the learning rate was decreased twice according to the scheduler used in CondInst (Tian et al. 2020).

Hardware and Software

We implemented our method using the AdelaiDet (Tian et al. 2019) and Detectron2 (Wu et al. 2019) frameworks and utilized the Augmentor Python library (Bloice et al. 2017) for pre-processing and, in particular, for generating elastic transformations. All our experiments were conducted on a workstation equipped with an AMD EPYC™ 7702P 64-Core CPU, 256 GB RAM, and four NVIDIA® A100-PCIe-80GB GPUs.

For runtime measurements, we used only a single GPU to run the instance segmentation model. The tile selection model was applied as a parallelized pre-processing step on the CPU.

Quality Metrics

We evaluated the EfficientNet approach for choosing countable image regions by using the accuracy score, a widely used metric defined as the proportion of true positive results, i.e., both true positives and true negatives in all predictions made by the model. Furthermore, we calculated the F1 score, which is the harmonic mean of recall and precision, i.e., $2 \cdot \frac{p \cdot r}{p+r}$. Recall and precision were computed as $p = \frac{\mathrm{TP}}{\mathrm{TP+FP}}$ and $r = \frac{\mathrm{TP}}{\mathrm{TP+FN}}$, where TP, TN, FP, and FN are true positives, true negatives, false positives, and false negatives, respectively. We evaluated our instance segmentation models in terms of average precision (AP), a common metric for object detection tasks. The AP is defined as the mean precision for equally spaced recall values. The metric corresponds to the area under the precision-recall curve, where predicted bounding boxes with an Intersection over Union (IoU) of more than a threshold t are considered to be true positives (TPs). For two sets of pixels, the IoU is defined as $IoU(A, B) = \frac{A \cap B}{A \cup B}$. Predictions that have no matching ground truth boxes are false positives (FPs) and ground truth boxes with no matching prediction are false negatives (FNs). For a given IoU threshold t, the AP is computed as an interpolation based on 101 values (Lin et al. 2014):

$$AP(t) = \frac{1}{101} \sum_{r \in \{0, 0.01, \ldots, 1\}} p_{interp}(r),$$

where $p_{interp}(r) = \max 1_{\tilde{r}:\tilde{r} \geq r}\, p(\tilde{r})$ and $p(\tilde{r})$ is the measured precision at recall $\tilde{r}$. In our experiments, we set t=0.5, i.e., an IoU of 50% between ground truth and the predicted bounding box or segmentation is required for a proposal to be counted as a true positive. We denote this metric as AP@50.

To evaluate the overall performance, we calculated the mean AP (mAP) score by taking the mean value of the AP scores from the different classes.

We performed each experiment five times with random seeds and report the standard deviation to make sure that we present reliable results.

3.5 Results

Tile Selection

The models were evaluated on a held-out dataset consisting of 298 positive and 346 negative examples.

Table 3.2: Results for tile selection model. We present accuracy and F1 score for the smallest available EfficientNet architectures, i.e., B0 and B1. All experiments were performed five times, and the standard deviation is reported. Values in bold indicate the best results for each metric.

Model	Accuracy	F1 Score
EfficientNet-B0	0.975 ± 0.002	0.973 ± 0.003
EfficientNet-B1	0.970 ± 0.006	0.968 ± 0.006

As Table 3.2 shows, both of the models performed very well with accuracies and F1 scores above 96%. The smaller version, i.e., EfficientNet-B0, performed better with an accuracy of 97.5% and an F1 score of 97.3%.

Detection and Segmentation

First, we performed the training with no augmentation at all. The results are summarized in Table 3.3 for the detection task. Adding the default data augmentation of CondInst, i.e., random horizontal flipping, improved the results by roughly 1.9% in terms of mAP. The application of further data augmentation, namely, random vertical flipping, random brightness, random contrast, and random saturation, again improved the score by roughly 2.8%. If we instead applied horizontal flipping and elastic deformations, the models still achieved a mAP of 87.7%. Combining all data augmentation methods in one model resulted in the best model, achieving 98.9% for erythrocytes, 90.2% for lymphocytes, 87.3% for eosinophils, and 86.3% for heterophils in terms of AP and, hence, a mAP score of 90.7%. Overall, combining all data augmentation methods resulted in an improvement of roughly 5.2% in terms of mAP.

Table 3.3: Detection results for erythrocytes and several subtypes of leukocytes. We experimented with different methods for data augmentation, i.e., random horizontal flipping (HFlip), random combinations of vertical flipping, brightness, contrast and saturation (DA), and elastic deformations (ED). We experimented with different architectures, i.e., CondInst and Mask R-CNN, and backbone models, i.e., ResNet-50 (R-50) and ResNet-101 (R-101). We report average precision at an Intersection over Union (IoU) of 50% (AP@50) for each class and the corresponding mean average precision (mAP). Values in bold indicate the best results for each metric.

Model	Backbone	HFlip	DA	ED	Detection (AP@50)				
					Erythrocyte	Lymphocyte	Eosinophil	Heterophil	mAP
CondInst	R-101				0.989 ± 0.000	0.847 ± 0.020	0.837 ± 0.006	0.765 ± 0.016	0.860
	R-101	✓			0.989 ± 0.000	0.869 ± 0.009	0.849 ± 0.007	0.799 ± 0.014	0.877
	R-101	✓	✓		0.989 ± 0.000	0.896 ± 0.004	0.869 ± 0.004	0.852 ± 0.009	0.902
	R-101	✓		✓	0.989 ± 0.000	0.882 ± 0.004	0.862 ± 0.008	0.813 ± 0.009	0.887
	R-101	✓	✓	✓	0.989 ± 0.000	$\mathbf{0.902} \pm 0.006$	$\mathbf{0.873} \pm 0.006$	$\mathbf{0.863} \pm 0.011$	**0.907**
CondInst	R-50	✓	✓	✓	0.989 ± 0.000	0.877 ± 0.007	0.853 ± 0.004	0.818 ± 0.007	0.884
Mask-RCNN	R-101	✓	✓	✓	0.938 ± 0.004	0.844 ± 0.006	0.785 ± 0.007	0.812 ± 0.005	0.845

Table 3.4: Instance segmentation results for erythrocytes and several subtypes of leukocytes. We experimented with different methods for data augmentation, i.e., random horizontal flipping (HFlip), random combinations of vertical flipping, brightness, contrast and saturation (DA), and elastic deformations (ED). We experimented with different architectures, i.e., CondInst and Mask R-CNN, and backbone models, i.e., ResNet-50 (R-50) and ResNet-101 (R-101). We report average precision at an IoU of 50% (AP@50) and the corresponding mean average precision (mAP). Values in bold indicate the best results for each metric.

Model	Backbone	HFlip	DA	ED	Segmentation (AP@50)				
					Erythrocyte	Lymphocyte	Eosinophil	Heterophil	mAP
CondInst	R-101				0.989 ± 0.000	0.846 ± 0.019	0.835 ± 0.006	0.766 ± 0.017	0.859
	R-101	✓			0.990 ± 0.000	0.871 ± 0.010	0.846 ± 0.007	0.798 ± 0.014	0.876
	R-101	✓	✓		0.990 ± 0.000	0.895 ± 0.004	0.869 ± 0.004	0.852 ± 0.009	0.902
	R-101	✓		✓	0.990 ± 0.000	0.883 ± 0.007	0.860 ± 0.008	0.812 ± 0.010	0.886
	R-101	✓	✓	✓	0.990 ± 0.000	$\mathbf{0.903} \pm 0.007$	$\mathbf{0.873} \pm 0.006$	$\mathbf{0.862} \pm 0.013$	**0.907**
CondInst	R-50	✓	✓	✓	0.990 ± 0.000	0.877 ± 0.009	0.853 ± 0.004	0.817 ± 0.006	0.884
Mask R-CNN	R-101	✓	✓	✓	0.938 ± 0.004	0.844 ± 0.006	0.783 ± 0.007	0.812 ± 0.005	0.844

The results of the instance segmentation shown in Table 3.4 are similar to those obtained for the corresponding bounding box detections. Each additional data augmentation step increased the mAP scores, and the best result was achieved by applying all random data augmentation techniques. However, the best model was not as dominant as in the detection task and could not outperform the other approaches in every category.

We observe that erythrocytes were continuously recognized almost perfectly with 98.9% and 99.0% AP for detection and segmentation, respectively. Thus, the model learned to not confuse thrombocytes or immature erythrocytes with erythrocytes. However, there was also an obvious drop in performance for all leukocyte subclasses. On the one hand, erythrocytes were easiest to identify because of the characteristic nucleus, and on the other hand, they were by far the most frequent cell type in avian blood samples, i.e., roughly 98% of all instances in our dataset. Therefore, the model could learn better features from this large set of samples. Among the leukocytes, this trend was evident as well. The most frequent leukocyte class, i.e., lymphocytes, still achieved 90.0% in terms of AP, while eosinophils and heterophils achieved 87.3% and 85.2%, respectively. Thus, there appears to be a correlation between the number of training samples and performance, which is, however, not statistically significant.

To further analyse the relation of precision and recall in more detail, we plotted the precision–recall curve of our best model in Figure 7. The graph of the erythrocyte (blue line) class is almost perfect, as expected, with an AP and, hence, an area under the curve of 99%. The graphs for the other classes start descending sooner, but by choosing the best threshold, in our case 0.5, a good balance between precision and recall could be achieved.

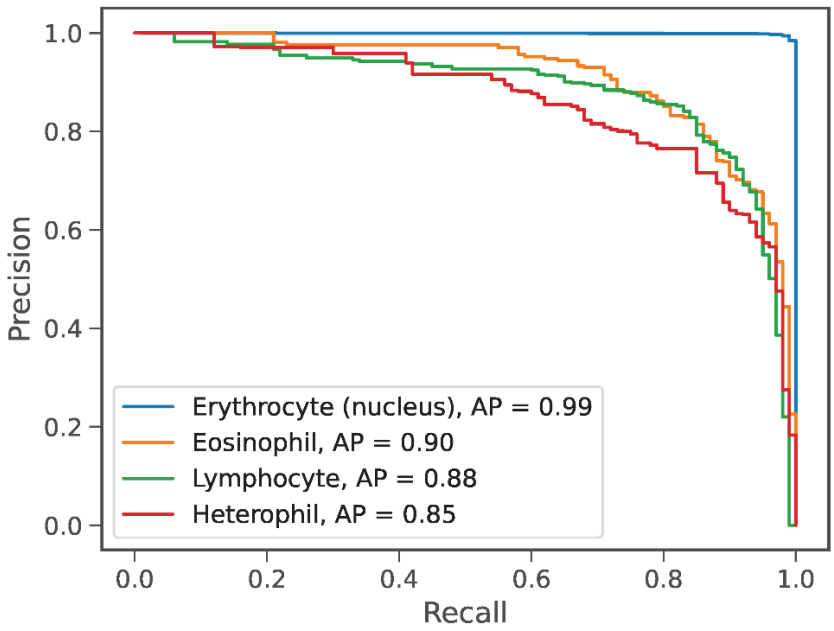

Figure 3.7: Precision–recall curve. The curves for the corresponding cell types are drawn in different colours, i.e., blue for erythrocytes, orange for eosinophils, green for lymphocytes, and red for heterophils.

CondInst with a smaller backbone, namely ResNet-50, performed very well but could not compete with the model based on ResNet-101. In comparison, the performance deteriorated by roughly 2.5% in terms of mAP. However, the anchor-based Mask R-CNN approach using a ResNet-101 backbone showed a clear drop in performance of roughly 6.8% compared to the anchor-free CondInst approach using the identical backbone.

Table 3.5: Detection results for binary model. We report the AP at an IoU of 50% (AP@50) based on the predicted bounding boxes for the CondInst architecture with two different backbone models, namely, ResNet-50 (R-50) and ResNet-101 (R-101). Values in bold indicate the best results for each metric.

Model	Backbone	Detection (AP@50)		
		Erythrocyte	Leukocyte	mAP
CondInst	R-50	0.989 ± 0.000	0.933 ± 0.002	0.961
	R-101	0.989 ± 0.000	0.936 ± 0.002	0.963

We did not have enough samples of basophils and monocytes for a comprehensive evaluation of their respective classes, but these samples could be aggregated into their superclass leukocytes. We trained a binary CondInst model that could classify avian blood cells into erythrocytes and leukocytes. As Table 3.5 and Table 3.6 show, our model could perform very well on this task, achieving more than 93% and 98.8% in terms of AP for leukocytes and erythrocytes, respectively. As before, the larger backbone, i.e., the ResNet-101, pushed the model to a better performance on leukocytes.

Table 3.6: Segmentation results for binary model. We report the AP at an IoU of 50% (AP@50) based on the predicted segmentation masks for the CondInst architecture with two different backbone models, namely, ResNet-50 (R-50) and ResNet-101 (R-101). Values in bold indicate the best results for each metric.

Model	Backbone	Segmentation (AP@50)		
		Erythrocyte	Leukocyte	mAP
CondInst	R-50	0.990 ± 0.000	0.932 ± 0.002	0.961
	R-101	0.990 ± 0.000	**0.937** ± 0.002	**0.964**

The mAP score regarding all leukocytes was higher than for any of the subclasses. Presumably, the multi-class model confused cell instances of the different subclasses.

Inference Runtimes

The inference runtimes for samples of different sizes are shown in Table 3.7.

Table 3.7: Segmentation results for binary model. We report the AP at an IoU of 50% (AP@50) based on the predicted segmentation masks for the CondInst architecture with two different backbone models, namely, ResNet-50 (R-50) and ResNet-101 (R-101). Values in bold indicate the best results for each metric.

Sample	Width (px)	Height (px)	Area (px)	Overall tiles	Countable tiles	Fraction (%)	Time (mm:ss)
1_023	195,126	91,974	17.947 B	91,059	49,369	54.22	52:01
1_044	155,375	92,066	14.305 B	72,417	61,998	85.61	57:07
2_013	63,743	65,396	4.169 B	21,080	4,140	19.64	05:26
5_055	53,784	46,887	2.511 B	12,705	535	4.21	01:58
8_036	205,167	93,585	19.201 B	97,200	24,743	25.46	25:09
8_040	195,216	88,232	17.224 B	87,249	9,697	11.11	13:39

We included the largest whole slide image (i.e., sample 8_036) consisting of more than 19 billion pixels as well as the smallest sample (5_055) with only roughly 2.5 billion pixels. However, in addition to the size of the image, the fraction of actual countable tiles played a crucial role in the processing times. For the largest file (8_036) containing 97,200 tiles with a countable tiles fraction of roughly one-fourth, our approach took roughly 25 min. Yet, another sample (1_023) with only 91,059 tiles, but more than half of them classified as countable, took roughly 52 min. Processing the three smaller samples took less than 15 min each. In general, none of the selected images needed more than one hour to determine the cell counts in the corresponding blood smear. Depending on the mentioned factors, processing took mostly less than a tenth of a second for one countable tile, including tile selection, segmentation, and identification, as well as counting of the respective cell instances. In contrast, our human expert took an average of roughly two minutes to annotate a tile with labels and segmentation masks in our semi-automated setting.

3.6 Discussion

Our novel approach offers a proficient assessment of avian blood scans, which speeds up the workflow of blood cell counting significantly compared to the traditional method of visually counting on microscopes. Compared to existing hardware devices for automated blood

analysis (Abbott; CellaVision), which are usually quite expensive, our approach is freely available. Hence, we enable researchers who do not have access to such devices used in veterinarian laboratories to utilize an automated cell-counting method. The CellaVision® DC-1 analyser has been evaluated for mammalian, reptilian, and avian blood by comparing its pre-classification to the final results after review by veterinarians (Leclerc 2021). The agreement was very good for neutrophils, heterophils, and lymphocytes (each > 90%) and good for monocytes (81%). However, eosinophils and basophils needed massive re-classification by human experts. Interestingly, while we agree that achieving good performance for basophils is a challenge, our model appears to be more reliable for eosinophils. However, we could not evaluate our model on monocytes that were recognized in a satisfactory way by the CellaVision® DC-1. Moreover, our approach can be more efficient than hardware-based approaches. The DC-1 (CellaVision) analyser processes given slides sequentially, achieving a throughput of no more than roughly 10 slides an hour. Our approach allows users to scan slides with various methods, e.g., with microscope cameras or high throughput scanners, like the Leica Aperio AT2 Scanner (Leica Biosystems) with a capacity of 400 slides, as used in our study. The Leica Aperio AT2 Scanner can be used to digitize a large number of slides in a very time-efficient manner. Our approach can be arbitrarily scaled by processing several slide images in parallel and is only limited by the available hardware resources. Furthermore, our approach can handle low-quality blood smears because it has been trained under such conditions, while the CellaVision® DC-1 analyser is primarily designed for usage in veterinary laboratories. Moreover, because of its proprietary design, it is not possible to use custom training data to adapt the classification approach. Hence, regarding large numbers of avian blood data sampled in ornithological field studies, our approach opens new possibilities for bird-related research.

While our approach shows that it is feasible to automatically count not only red but also white avian blood cells with open-source software, it still has some downsides. Because of the low number of samples in our training set, our neural network model is not yet able to reliably recognize basophils or monocytes. Furthermore, the model is trained on a limited number of bird species. Because of potential variations in staining intensity, colouration, and cell morphology, it may be a challenge to detect cells of other bird species as reliably as for the given species (Feldman et al. 2000). In particular, eosinophils may be quite different between bird species.

However, these issues indicate several areas for future work. The model performance can be further improved by extending the dataset in general and particularly for the rare classes, i.e., for basophils and monocytes. Instead of bluntly annotating more images that barely contain any of these cells, this can be done using an active learning approach, which reliably provides unlabelled images that contain these types of avian white blood cells. Moreover, it is a promising direction to generate more training samples by generative deep learning approaches, like GANs (Goodfellow et al. 2020), or image generation models based on latent diffusion (Rombach et al. 2022). Furthermore, our approach can be extended to recognize and count blood parasites (e.g., Haemosporida and Trypanosoma). Another interesting aspect is investigating and improving the generalization ability of our neural network model in cross-domain scenarios. This can include different techniques when creating blood smears for different bird species. We plan to include further bird species into our model, e.g., penguins.

Several studies have indicated that extreme ecological conditions can significantly increase haematocrit levels in birds. For example, a female great tit from the northernmost populations in Northern Finland showed a haematocrit level of 0.83 (Krama et al. 2013). This makes the blood viscous and leads to densely packed cells in the blood smear image, which can be challenging for automated counting approaches. Since we trained our model to count only areas matching human quality standards, we only counted tiles from the monolayer. Hence, a high haematocrit level may lead to significantly more rejected tiles. However, our approach is adaptable to new annotated data sources. Thus, providing our models with manually labelled images with high haematocrit levels in future training iterations will improve their ability to process and count cells with such rare conditions better. In general, our approach is based on open-source software. Therefore, the models can easily be adapted to other datasets or extended to recognize further cell types.

So far, our approach aims to automate the tedious task of manually counting avian blood cells. Furthermore, it eliminates inter-observer errors. However, it still counts cells only in the monolayer. Future work may expand the countable areas, as achieved by handcrafted feature algorithms (Govind et al. 2018). For a deep learning approach like ours, this can be achieved by training the model with data involving lower-quality areas. By learning useful features from the annotated samples, the resulting models may be capable of achieving superhuman performance.

Our deep learning model opens up new opportunities in ornithology and ecology for documenting and evaluating the stress levels and health conditions of bird populations and communities efficiently and can, therefore, be used as an early warning indicator to detect physiological changes within populations or communities even before a population decline. With this fast, reliable, and automated approach, even old collection samples may retrospectively be incorporated into modern ornithological research. Our approach is currently used in practice for research on the relative stress load of forest birds by automatically determining H/L ratios.

3.7 Conclusions

We presented a fully automated open-source software approach to determine not only the total erythrocyte count but also the total and differentiated leukocyte counts of avian blood samples. Our approach operates on whole slide blood smear images. First, we select tiles using a deep neural network model to determine the areas of the images that are suitable for counting contained cells by classifying all tiles into countable and non-countable ones. Each tile classified as countable is then fed into another deep neural network model. This model is capable of detecting and classifying avian blood cells, i.e., erythrocytes and leukocytes, with 96.1% in terms of mAP. Furthermore, if the model is trained to also recognize subtypes of leukocytes, it achieves up to 98.9%, 90.2%, 87.3%, and 86.3% in terms of AP for erythrocytes (with nuclei), lymphocytes, eosinophils, and heterophils, respectively.

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Author contributions

Conceptualization, M.V. and F.S.; methodology, M.V., F.S., H.B., M.M., N.K. and D.S.; software, M.V.; validation, M.V.; investigation, M.V. and F.S.; resources, F.S., S.R. and D.G.S.; data curation, M.V. and F.S.; writing—original draft preparation, M.V. and F.S.; writing—review and editing, all authors; visualization, M.V.; supervision, M.M., N.F., D.G.S., S.R. and B.F.; project administration, N.F. and B.F.; funding acquisition, N.F. and B.F.; All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

Data are contained within the article. All relevant software and data presented in this article, including the trained neural network models, are openly available at https://data.uni-marburg.de/handle/dataumr/250 (accessed on 27 February 2024). Furthermore, our software repository is publicly available at https://github.com/umr-ds/avibloodcount (accessed on 27 February 2024).

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Ethics statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Regional Council of Giessen, Hesse, Germany (protocol code V 54–19 c 20 15 h 01 MR 20/15 Nr. G 10/2019 and date of approval 26 February 2019).

Chapter IV: Intrinsic factors influence physiological stress in a forest bird community: Adults and females have higher H/L ratios than juveniles and males.

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4.1 Abstract

Physiological parameters have the potential to serve as valuable early warning indicators for the conservation of animal populations. However, measuring physiological adaptations in wildlife is often challenging, due to short time windows for sampling or the lack of knowledge about baseline levels depending on the chosen method. Furthermore, intrinsic differences cause natural variation of physiological measures between individuals across species. This study is aimed at filling this gap by investigating the influence of intrinsic factors, including sex, age, body condition, and the incubation of eggs on the H/L ratio of a forest bird community. As physiological measure, we used the heterophil to lymphocyte (H/L) ratio of the bird community which was assessed using a novel deep learning approach based on Convolutional Neural Networks applied to whole blood smear scans. Using phylogenetically controlled analyses across the bird species, we found higher H/L ratios in adult birds than in juveniles and observed higher stress levels in females than in males. While body condition had no effect on the H/L ratio, incubating birds tended to have higher H/L ratios than non-reproductive birds, regardless of their sex. Furthermore, we found a robust phylogenetic signal of the H/L ratio in the studied bird community. Our results reveal significant general patterns of the effect of intrinsic factors on the H/L ratio across a bird community.

4.2 Introduction

Birds are one of the most extensively researched animal groups, with long-term population dynamics data available for most species across a wide geographical range (Møller et al. 2010).

Over the last few centuries, bird populations have declined in both abundance and biomass, with common bird species being particularly affected by this loss (Inger et al. 2015; Rosenberg et al. 2019). Even before populations decline, individuals show physiological adaptations in response to the threats causing the decline (Wikelski und Cooke 2006; Dantzer et al. 2014; Seebacher et al. 2023). Therefore, measuring physiological parameters can help to identify threats and to develop suitable conservation measures (Wikelski und Cooke 2006; Dantzer et al. 2014; Seebacher et al. 2023). However, such physiological measures do not only vary due to environmental factors and stressors but also vary naturally throughout the year and due to other intrinsic factors, such as sex and age (Hõrak et al. 1998; Campo und Davila 2002; Kilgas et al. 2006; Jakubas et al. 2011; Vincze et al. 2022). Therefore, when interpreting physiological measures, it is crucial to first understand the influence of intrinsic factors on the chosen measure within the studied sample of birds from a certain population or community. Physiological adaptations however come with a cost as usually more energy is allocated towards stress reactions and ad hoc survival which can on the long-term cause lower fitness and a declining physical state at the individual level, and ultimately contributing to a decline in the population (Moberg 2000; Harris et al. 2002; Cyr und Romero 2007). Consequently, measuring stress in wildlife can be used as an early warning indicator for population declines and changes in community structures and even to evaluate habitat quality, resource availability, and ecosystem health (Wikelski und Cooke 2006; Davis et al. 2008).

In practice, measuring and interpreting physiological measures in wildlife poses substantial challenges due to short time windows for sampling and the lack of information on baseline levels and comparable parameters of conspecifics (Davis et al. 2008; Sheriff et al. 2011; Johnstone et al. 2012). In birds, stress physiology is comparably well studied and frequently chosen as physiological measure (Breuner et al. 2003; Davis et al. 2008; Almasi et al. 2010; Homberger et al. 2013; Catitti et al. 2022). In studies focusing on poultry and captive birds, baseline stress levels can be easily acquired (Davis et al. 2008). In contrast, in a natural environment, measuring and interpreting stress levels of birds can be difficult due to the necessity of quick sample collection or a lack of baseline stress levels and the regular presence of varying environmental stressors and variation of the stress levels due to natural fluctuations of the respective stress metric (Vleck et al. 2000; Davis et al. 2008).

Numerous studies, both in captivity and in the wild, have shown that in addition to the stress response, stress levels can also fluctuate due to a number of intrinsic factors such as age, sex, body condition, and reproductive status of the respective individuals (Campbell und Dein 1984; Dantzer et al. 2014; Tablado und Jenni 2017). When investigating the seasonal variation of stress hormones (glucocorticoids), it becomes evident that in free living reptiles, amphibians and birds, stress levels are commonly higher during the breeding period compared to both pre-breeding and post-breeding periods (Romero 2002). Studies focusing on single species however present inconsistent results regarding the influence of specific intrinsic factors (Hõrak et al. 1998; Jakubas et al. 2011; Tablado und Jenni 2017; Skwarska 2019). For example, age was found to have a significant effect on stress levels, but the direction of this effect is inconsistent. In Spanish chicken (Quail Castellana), juveniles were found to have lower stress levels than adults (Campo und Davila 2002). However, a study on reed buntings (*Emberiza schoeniclus*) found that immature birds had higher stress levels than adults (Jakubas et al. 2011). Sex and Season did in the same study on the contrary not affect the stress levels (Jakubas et al. 2011). Several further studies however detected fluctuations of the stress levels driven by sex. A study on great tits (*Parus major*) for example found that breeding females showed higher stress levels than males (Hõrak et al. 1998; Kilgas et al. 2006). However, this effect was only found during the breeding period. Prior to the start of the breeding period, males exhibited higher stress levels than females (Hõrak et al. 1998). A study on red-footed boobies (*Sula sula*) revealed a rise of stress levels during egg-incubation and brooding of thermally dependent nestlings. Afterwards, during the brood rearing, stress levels started to decrease again. The pattern was similar for males and females, yet more pronounced in females (Lormée et al. 2003). Apart from sex, the overall body condition was found to affect the stress physiology in both adult and juvenile birds. Stress levels were found to be higher in birds with a worse body condition during migration than in their conspecifics with better body condition (Włodarczyk et al. 2018). Similar results have also been reported from other studies, suggesting that birds in a poor body condition are experiencing stress (Gladbach et al. 2010). Previous studies and reviews have discussed the complexity of stress response variation driven by intrinsic and environmental factors and the importance to consider these factors when analysing stress in wildlife populations (Breuner et al. 2003; Almasi et al. 2010; Homberger et al. 2013; Dantzer et al. 2014; Tablado und Jenni 2017; Catitti et al. 2022). However, the consistency of stress variation due to intrinsic factors across a bird community

has not yet been analysed. To the best of our knowledge, the variance of the measured stress levels caused by intrinsic factors has only been investigated in a single species (Campo und Davila 2002; Jakubas et al. 2011; Cīrule et al. 2012; Frigerio et al. 2017; Gao et al. 2017; Włodarczyk et al. 2018). However, it has not yet been investigated whether the effect of these intrinsic factors on a chosen stress measure is consistent across a bird community, thus allowing for a cross-species and community-wide analysis of stress measures. This may be due to species-specific differences in stress response that make direct comparisons difficult. For example, species may have different immune functions depending on their social behavior and whether or not they migrate (Côté und Poulin 1995; Møller und Erritzøe 1998; Lindström et al. 2004; Davis 2012; Pap et al. 2015; Minias et al. 2017; Minias et al. 2018). Furthermore, there is evidence for a trade-off between immunity and reproduction, leading to a weaker immunity in birds with larger clutch sizes and higher parental investment (Norris und Evams 2000; Arida 2005; Hanssen et al. 2005; Knowles et al. 2009). To accurately evaluate differences in stress response both within and across species, it is necessary to have a comprehensive understanding of the impact of intrinsic factors on stress and comparative data that enables appropriate interpretation of the results.

When applying wildlife stress physiology in bird communities, it is crucial to select a feasible measurement method. Various methods are available for measuring bird stress, including the assessment of plasma corticosterone levels (Jenni-Eiermann et al. 2008; Sheriff et al. 2011; O'Dell et al. 2014) or fecal corticosterone metabolites (Davis et al. 2008; Sheriff et al. 2011; Rösner et al. 2023), by evaluating leukocyte counts (Davis et al. 2008; Davis und Maney 2018) and calculating the ratio of heterophils to lymphocytes (H/L ratio; Davis et al. 2008; O'Dell et al. 2014; Davis und Maney 2018) or by assessing the levels of heat shock proteins (O'Dell et al. 2014). Each of these methods has advantages and challenges (Johnstone et al. 2012). When selecting an appropriate stress measure, it is important to consider both the handling time and costs, as well as the different potential stressors influencing stress levels and the physiological functioning as different stress measures not necessarily reflect the same stress (Müller et al. 2011). While measuring plasma corticosterone requires sampling within three minutes after capture and cost-intensive laboratory work (Pereyra und Wingfield 2003; Jenni-Eiermann et al. 2008; Sheriff et al. 2011; Davis und Maney 2018), fecal corticosterone sampling has the advantage of being non-invasive for the individuals but is often linked to time-consuming field and lab work (Sheriff et al. 2011; Rösner et al. 2023). In contrast to these

methods, using leukocyte profiles combines the advantages of being cost efficient and not requiring immediate sampling, since changes are detectable only 30-60 minutes after capture (Davis et al. 2008; Cīrule et al. 2012). Various hematological parameters can be used to evaluate the bird's condition. One such parameter is the H/L ratio, which is known to indicate stress levels resulting from intrinsic and environmental stressors (Davis et al. 2008). However, the H/L ratio does not solely reflect the stress level of birds, but also changes in response to parasites, infections, and different life stages, as well as being dependent on the ontogeny of the birds. This aspect needs to be considered when interpreting and discussing the results. Changes in the H/L ratio in response to stress are triggered by elevated glucocorticoid levels which trigger an influx of heterophils into the bloodstream, while lymphocytes undergo redistribution to other tissues (Siegel 1980; Gross und Siegel 1983; Davis et al. 2008). Consequently, the H/L ratio can serve as an indicator of stress in birds. Counting leukocytes manually to evaluate blood smears however requires expertise and is time-consuming. Thus, automated blood cell counting, as that developed for human blood, potentially increases the attractiveness of this method for assessing wildlife stress in bird communities (Ramesh et al. 2012; Walliander et al. 2013).

The aim of our study was to detect general patterns of the influence of intrinsic factors on the H/L ratio across multiple species of a forest bird community. To advance the method for application in wildlife stress assessments, we used a novel deep learning approach based on a Convolutional Neural Network (CNN) for automated cell detection and counting in digitized images of stained blood smears (Vogelbacher et al. 2024). Since the H/L ratio varies among bird species and shows a relatively strong phylogenetic signal, we controlled for phylogenetic relationships in stress levels in our analyses (Davis 2012; Minias 2019). Considering these phylogenetic relationships, we hypothesize that (1) adult birds have higher H/L ratios than juveniles due to their experience and costs of parental care, (2) females exhibit higher H/L ratio than males as found by previous studies during the breeding season, (3) birds with poorer body condition display a higher H/L ratio than birds with good body condition, (4) birds incubating eggs show a higher H/L ratio than non-incubating birds, and (5) that there is a phylogenetic signal in the H/L ratio of the species in our bird community. By exploring these hypotheses, this study aims to detect general patterns of the influence of intrinsic factors on the H/L ratio across a forest bird community during the breeding period in spring and summer. Identifying these patterns will help to assess the feasibility of using the H/L ratio across

species, which would allow future comparisons of the effects of general environmental or habitat changes on whole bird communities.

4.3 Material and Methods

Study site and bird capture

We conducted our study in the Marburg Open Forest, a 250-hectare beech (*Fagus sylvatica*) - dominated managed temperate forest ecosystem in Hesse, Germany. Data collection took place over four consecutive years, from 2019 to 2022. Birds were captured during the breeding seasons between mid-March and August at ten sites in the forest interior. When selecting the mist-netting sites, we aimed to cover as broad a spectrum of habitat and age structures in the forest as possible. In 2019, we conducted three sampling rounds, resulting in 30 capture events. From 2020 onwards, we increased the sampling effort to six rounds, resulting in a total of 60 capture events. At each site, we set up a row of three mist nets, each 12 meters long, totalling 36 meters of net. Nets measured 2.5 meters in height with a mesh size of 16 mm x 16 mm. Starting from 2020, every other capture event at each site was conducted with high nets measuring five meters in height. No lures were used to attract the birds to the nets to avoid artificially influencing their natural movement or stress levels. The mist nets were checked every 15 to 20 minutes. Upon capture, each bird was marked with a ring for future identification (e.g., re-captures), with the necessary permissions obtained from the Heligoland Bird Observatory (Institute of Avian Research "Vogelwarte Helgoland" (IAR), Germany).

Morphological measurements and sample collection

We measured various morphological characteristics of each captured bird, including wing length, length of the third outermost primary (P8), and weight. We calculated the body condition of each bird by dividing the weight by the length of the third outermost primary feather (P8; Johnson et al. 1985; van Balen 2002). Based on that we calculated the body condition index by multiplying the individual body condition with 100 divided by the mean body condition of the species ($BC_{ind} \times \frac{100}{BC_{mean}}$) to account for species specific differences. Values below 100 indicate a below-average body condition, while values above 100 indicate

an above-average body condition for the respective species in our forest bird community. We chose P8 length over wing length due to its reduced susceptibility to measurement errors (Berthold und Friedrich 1979). Wherever possible, we determined the age and sex of the bird based on its moult pattern and morphology. We determined birds hatched in the respective sampling year as juvenile and birds in their second year or older as adult. Furthermore, we examined the bird for the presence of a brood patch to gather information on whether it is involved in incubating eggs or not.

Blood samples were taken by puncturing the brachial vein and using heparinized capillary tubes, following the animal testing approval granted by the regional council of Giessen, Hesse, Germany (V 54–19 c 20 15 h 01 MR 20/15 Nr. G 10/2019). The H/L ratio only rises 30 to 60 minutes after a stressful event (Davis 2005; Cīrule et al. 2012; Davis und Maney 2018). To prevent handling stresses from influencing the extracted stress values, all blood samples used for stress analysis were taken within 30 minutes after capturing, directly after extracting the bird from the net (Davis 2005; Cīrule et al. 2012). We prepared whole blood air dry smears for microscopy using the standard two-slide wedge procedure (Ruiz et al. 2002) and preserved blood on Whatman FTA Classic cards (Whatman, UK) for DNA analysis.

Laboratory work

For specimens in which field-based sex determination was not feasible due to the absence of sexual dimorphism, we utilized genetic sexing techniques. DNA extraction and isolation were performed using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). A PCR was carried out using the primer pair P2/P8 (Griffiths et al. 1998) for sexing, and both positive and negative controls were included for each PCR run.

For assessing the relative stress response of the captured birds, we used whole blood air-dry smears (Ruiz et al. 2002). The blood smears were fixed in methanol and treated with Giemsa stain. To determine the H/L ratio, we digitized the blood smears by scanning them with a Leica Aperio AT2 Scanner and a 40X microscope objective lens.

Automated blood cell counts

To be able to automatically count the entirety of cells in a blood smear in adequate time, we used deep learning methods, in particular Convolutional Neural Networks (CNNs; LeCun et al. 2015; Vogelbacher et al. 2024). These models belong to the state-of-the-art in computer vision

and are competitive with more recent approaches (Liu et al. 2022), e.g., vision transformers (Dosovitskiy et al. 2020), in several applications. CNNs achieved excellent results in many biological and medical image tasks (Korfhage et al. 2020; Cheuque et al. 2022).

The scans generated throughout the study have large dimensions. They range from 47,807 to 205,176 px in width and from 35,045 px to 93,827 px in height. To be able to process these scans, we cropped them into tiles using the OpenSlide library (Goode et al. 2013). To analyse the tiles, we developed an approach based on two deep learning (i.e., artificial neural network) models. The first model decides for each tile of a scan whether it is countable or not. The second model detects and classifies the cells contained in the tiles that were classified as countable. Both models are briefly described below, for more detail see Vogelbacher et al. (2024).

The first neural network model, an EfficientNet-B0 (Tan und Le 2019) was trained to categorize each tile as either countable or non-countable. The criteria for training included having a single cell layer with evenly distributed cells across the image, good visibility of cells without significant amounts of dirt or stain remnants, and a good focus of the cells.

In a pre-processing step, we randomly applied data augmentation, including random contrast, random hue, random cropping, horizontal as well as vertical flipping, and elastic transformations. We optimized the pre-trained EfficientNet-B0 model for an overall of 50 epochs and selected the best checkpoint for the first model. Our model achieves an accuracy of roughly 98% and an F1-score of roughly 97%.

All tiles recognized as countable regions are then passed to our second neural network model for the detection and segmentation of the cells contained in the tiles. The task of this second neural network model is to detect each cell in these tiles and assign its specific cell type to it to be correctly counted. In the initial step, we manually assigned to each cell (erythrocytes as well as leukocytes) a segmentation mask indicating its exact boundaries and a corresponding class label, i.e. their respective cell types. Erythrocytes were labelled as such, while leukocytes were further categorized as lymphocytes, heterophils, eosinophils, basophils, or monocytes. Only cells with at least half of their surface visible were considered. The annotation process was then carried out using an iterative, model-assisted manner by selecting tiles with a high probability of having rare cell types. Overall, we conducted four iterations of labelling. All labelling was performed by a single expert (FS) to minimize the inter-observer bias. The

annotation effort resulted in a training dataset consisting of 1,450 images randomly selected from our bird community. These images showed 177,324 instances of erythrocytes, 1,504 lymphocytes, 898 eosinophils, 815 heterophils, 141 basophils, and 65 monocytes.

We trained the second neural network model based on this data set. We use the instance segmentation network CondInst (Tian et al. 2020) with a ResNet-101 (He et al. 2016) backbone as our neural network architecture and optimized the pre-trained network for more than 50 epochs. To further enrich the data set, and in particular the variety of the leukocyte classes, we applied extensive data augmentation. For this purpose, we used random vertical and horizontal flipping, random hue, brightness, and saturation as well as elastic transformations.

Our instance segmentation network achieved an average precision (AP) of 99% for erythrocytes, 86% for heterophils, 87% for eosinophils, and 90% for lymphocytes at an Intersection over Union (IoU; Rezatofighi et al. 2019) of 50% on a held-out test set. Due to the low number of samples, basophils and monocytes were not evaluated.

Applying our two trained neural networks in inference mode to count the cells in a given whole-slide image was straightforward. First, we cropped the image into smaller tiles. Each tile was fed into our fine-tuned EfficientNet-B0 determining whether the tile was countable or not. We set the score threshold to 70% which slightly favors precision over recall. Next, the images classified as countable were fed into our trained instance segmentation model based on CondInst with a ResNet-101 backbone. The number of cells for each type could then be inferred from the number of instances provided in the model outputs. However, it is important to filter for instances with low scores since these are not reliable. We determined that setting the threshold at 50% optimized the balance between precision and recall, ensuring an optimal performance.

Data analysis

For the data analysis, recaptures within one breeding season were removed (n=39). Recaptures of individuals across years were kept in the final dataset (n=30). We further removed birds with less than 300 countable tiles being found by the neural network (n=47) to ensure a good quality of the stress data as well as birds with fewer than five captured birds per family (n=6). Individuals with unknown sex (n=3) or body condition (n=17) were also removed.

The final dataset consisted of 409 birds from 22 species. For each of these birds, we determined whether the bird was potentially incubating based on the factors sex, age, species, and date. Information regarding the incubating sex and the breeding period were obtained from literature sources as summarized in Bezzel (1993). This allowed us to differentiate between birds that were potentially incubating but had not developed a brood patch and therefore were likely not incubating during the respective breeding season versus birds with a brood patch that were incubating eggs. Since the distribution of the resulting H/L ratio data exhibited an approximate exponential distribution, we applied a log transformation to achieve a normal distribution.

All statistical analyses were performed using the R software (R Core Team 2023). To test our first four hypotheses, we employed phylogenetic generalized linear mixed-effect models (PGLMM) using the phyr package (Li et al. 2020).

To incorporate phylogenetic information in our analyses, we obtained 1,000 phylogenetic trees for the species within our forest bird community using the birdtree website (https://birdtree.org; Rubolini et al. 2015). From this set, we selected the tree with the maximum clade credibility for further analysis. For testing our first hypothesis, we were using the mean logarithmized H/L ratio of each species and assessed the phylogenetic signal to determine whether to utilize a phylogenetic linear model or a normal linear model in subsequent analyses. To use the phylogenetic tree along with our H/L ratio on individual basis, we added tips for each individual to the phylogenetic tree with a phylogenetic distance of zero. Thereby, we were able to match phylogenetic information to each individual data in our dataset.

We tested for the impact of sex, age, and body condition on the H/L ratio by fitting a PGLMM. This model included sex, age, body condition index, day of the year, and year as independent variables and added the capturing site, bird species and Ring ID of the bird as a random effect. We included day of the year and year to account for seasonal and annual variation of environmental stressors, Ring ID to account for individual patterns of interannual recaptures and bird species to account for additional species-specific differences beyond phylogeny, such as variations in habitat preferences. During model fitting, all possible two-way interactions between predictors were incorporated into the full model. Subsequently, considering the lack of significance of any interactions ($\alpha = 0.05$), we proceeded to utilize all predictors as

independent variables. Additionally, in our modeling approach, we considered the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). Furthermore, we conducted model diagnostics using the DHARMa package (Hartig 2022) to ensure the validity of our modeling decisions. For the second model, testing the effect of body condition and reproductive status, we focused exclusively on potentially incubating birds, determined by the previously described criteria and again dropped birds with less than five individuals per family (n=152, 19 species). This model incorporated actual incubation status (presence or absence of a brood patch), sex, body condition index, day of the year and year as independent variables and capturing site, Ring ID and species as random effects. As in the first model, all possible two-way interactions were tested, however no significant interaction was found. We applied the same methods as in our first model for model fitting. Consequently, the final model included sex, brood patch, body condition index, day of the year and year as independent fixed effects and capturing site, Ring ID and species as a random effect.

4.4 Results

Across four breeding seasons, we caught 529 birds of 29 species, of which 409 birds of 22 species were included in the final data set, based on quality of blood smears and completeness of data on age, sex and body condition. The overall mean H/L ratio across species was 0.276 and ranged from 0.004 to 4.626 (Table 4.1), the highest H/L ratio was documented in *Dendrocopos medius* whereas *Turdus philomelos* had the lowest mean H/L ratio (Table 4.1).

Table 4.1: Summary of sampled bird specimen (N) per species and family. Mean H/L ratio along with the minimum (H/L min) and maximum (H/L max) H/L ratios observed are reported. The number of males (M), females (F), adult birds (Adult) and juvenile birds are given. 'PI' refers to 'Potentially Incubating' which was determined based on sex, age and capture date of the respective individuum. 'RS' refers to 'Reproductive Status,' indicating the number of birds with documented brood patches, suggesting incubation of eggs.

	N	H/L mean	H/L min	H/L max	M	F	Adult	Juvenile	PI	RS
Picidae	13	0.612	0.122	2.088	3	10	10	3	10	9
Dryocopus martius	1	0.520	0.520	0.520	0	1	1	0	1	1
Dendrocopos major	11	0.569	0.122	2.088	3	8	8	3	8	7
Dendrocopos medius	1	1.173	1.173	1.173	0	1	1	0	1	1
Paridae	120	0.309	0.004	4.626	61	59	101	19	45	39
Parus major	60	0.295	0.026	1.106	31	29	50	10	22	22
Poecile palustris	27	0.237	0.004	0.544	11	16	21	6	11	9
Lophophanes cristatus	5	0.244	0.168	0.372	4	1	3	2	1	1
Periparus ater	3	0.336	0.196	0.577	1	2	3	0	2	2
Cyanistes caeruleus	25	0.429	0.033	4.626	14	11	24	1	11	5
Sylviidae	57	0.342	0.03	1.663	34	23	56	1	49	45
Sylvia atricapilla	57	0.342	0.03	1.663	34	23	56	1	49	45
Phylloscopidae	17	0.191	0.034	0.517	9	8	16	1	8	5
Phylloscopus sibilatrix	6	0.146	0.112	0.214	3	3	6	0	3	0
Phylloscopus collybita	11	0.215	0.034	0.517	6	5	10	1	5	5
Certhiidae	19	0.196	0.058	0.600	16	3	13	6	3	3
Certhia familiaris	16	0.199	0.058	0.600	13	3	11	5	3	3
Certhia brachydactyla	3	0.180	0.066	0.288	3	0	2	1	0	0
Troglodytidae	11	0.301	0.059	0.692	9	2	7	4	0	0
Troglodytes troglodytes	11	0.301	0.059	0.692	9	2	7	4	0	0
Sittidae	17	0.412	0.11	1.941	11	6	12	5	3	3
Sitta europaea	17	0.412	0.117	1.941	11	6	12	5	3	3
Turdidae	52	0.156	0.020	0.383	39	13	51	1	13	13
Turdus viscivorus	1	0.219	0.219	0.219	1	0	1	0	1	1
Turdus philomelos	15	0.138	0.052	0.329	9	6	14	1	5	5
Turdus merula	36	0.163	0.020	0.383	29	7	36	0	7	7
Muscicapidae	71	0.170	0.026	0.937	48	23	57	14	16	16
Erithacus rubecula	71	0.170	0.026	0.937	48	23	57	14	16	16
Fringillidae	32	0.338	0.036	1.193	22	10	32	0	10	8
Coccothraustes coccothraustes	3	0.661	0.463	0.8	2	1	3	0	1	1
Pyrhula pyrrhula	3	0.189	0.111	0.283	2	1	3	0	1	1
Fringilla coelebs	26	0.317	0.036	1.193	18	8	26	0	8	6

When testing the effect of age, sex, and body condition on the H/L ratio for the dataset, we found that adults had a significantly higher H/L ratio than juveniles and that males exhibited lower H/L ratios than females (Table 4.2, Figure 4.1 & 4.2). Body condition had no significant effect on the H/L ratio of the birds (p = 0.179).

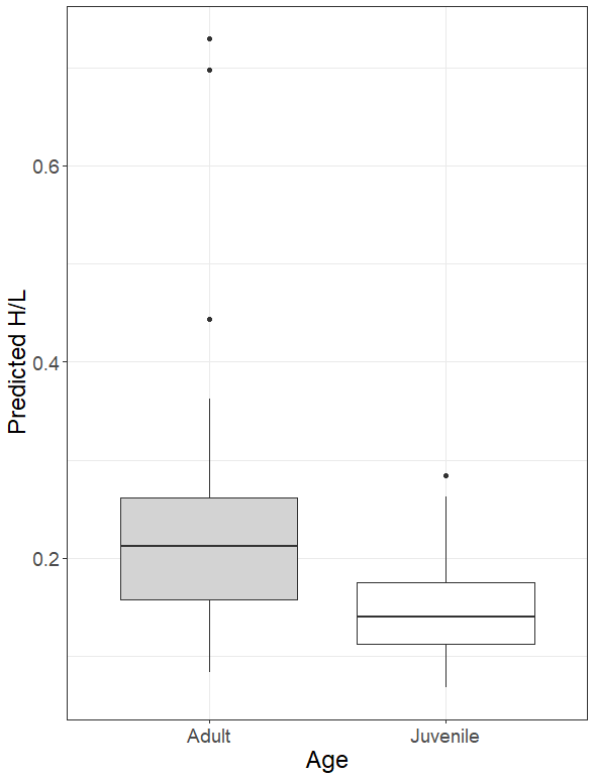

Figure 4.1: The predicted H/L values of the PGLMM showing age specific differences. Adults are displayed in grey, whereas juveniles are shown in white.

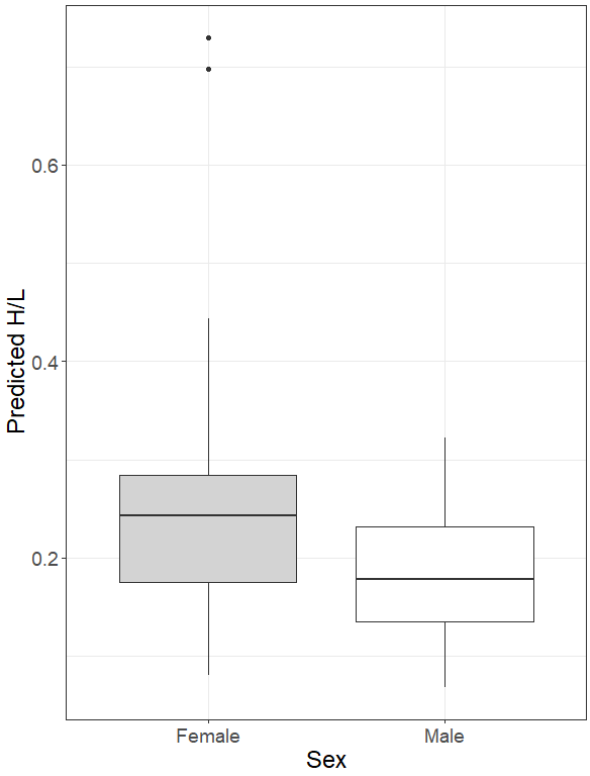

Figure 4.2: The predicted H/L values of the PGLMM showing sex specific differences. Females are displayed in grey, whereas males are shown in white.

Table 4.2: Results of the PGLMM testing the effect of sex, age, body condition, day of the year and year on the H/L ratio.

Fixed effect	Estimate	Standard error	Z-Score	P-Value
Intercept	-0.771	0.068	-11.281	< 0.001 ***
Sex (Ref: Female)				
Male	-0.076	0.036	-2.130	0.033 *
Age (Ref: Adult)				
Juvenile	-0.130	0.059	-2.217	0.027 *
Body condition	0.014	0.017	0.817	0.414
Day of the year	-0.006	0.021	-0.293	0.770
Year (Ref: 2019)				
2020	0.197	0.060	3.274	0.001 **
2021	0.157	0.061	2.563	0.010 *
2022	0.149	0.062	2.394	0.017 *

In our second model we only included potentially incubating birds in our dataset (n = 167). In the model we included sex, body condition and incubation status (brood patch yes or no) and found a marginal effect of incubation status with incubating birds having marginally significantly higher H/L ratios than non-incubating birds (Figure 4.3). Sex and body condition did not affect the H/L ratio (Table 4.3).

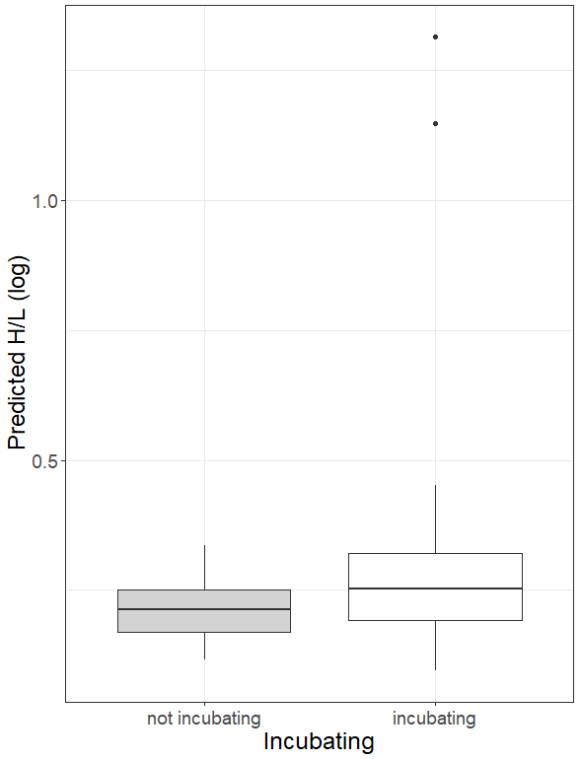

Figure 3: The predicted H/L values of the second model on potentially incubating birds. Not incubating birds are displayed in grey, incubating birds in white.

Table 4.3: Results of the second PGLMM: sex, body condition, incubating (yes or no), Day of the year and year on the H/L ratio of potentially incubating birds (adult and belonging to the breeding sex of this species)

Fixed effect	Estimate	Standard error	Z-Score	P-Value
Intercept	-0.946	0.140	-6.765	< 0.001 ***
Sex (Ref: Female)				
Male	-0.114	0.	-1.161	0.246
Incubating (Ref: No)				
Yes	0.152	0.086	1.770	0.077 .
Body condition	0.020	0.033	0.587	0.557
Day of the year	-0.036	0.043	-0.849	0.396
Year (Ref: 2019)				
2020	0.293	0.119	2.468	0.014 *
2021	0.207	0.120	1.726	0.084 .
2022	0.218	0.126	1.134	0.083 :

Overall, we found a significant phylogenetic signal of the H/L ratio across our bird community ($K = 0.799$, $p = 0.031$; $\lambda = 0.647$). Woodpeckers (*Picidae*) were found to have the highest mean H/L ratio (0.61), whereas the mean H/L ratio was lowest in thrushes (*Turdidae*, 0.16) and flycatchers (*Muscicapidae*, 0.17).

4.5 Discussion

Investigating the effect of intrinsic factors on the H/L ratio in a bird community, we found that adult birds and females had a significantly higher H/L ratio than juveniles and males across bird species during the breeding period. Additionally, we noted a slight influence of incubation status on the measured H/L ratio, with incubating birds showing marginally higher ratios than non-incubating birds. We also found a significant phylogenetic signal of the mean H/L ratio across the species of our bird community.

The influence of age, sex and breeding status on the H/L ratio

In line with our prediction, we found that adult birds had a higher H/L ratio compared to juvenile birds. This observation could be attributed to the stress load during parental care experienced by adults. Throughout the breeding season, adult birds adjust their behavior to promote the welfare of their offspring and increase the likelihood of successful reproduction (Biermann und Robertson 1981; Gow und Wiebe 2014). Although the costs of parental care remain incompletely understood (Santos und Nakagawa 2012), research has demonstrated that birds incubating eggs decrease their activity in the presence of predators to minimize the possibility of predation (Kovařík und Pavel 2011). Thus, the inhibition of the urge to fly during the breeding season, coupled with the additional cost of parental care, may contribute to increased stress levels in adult birds. This behavioral adaptation to breeding could potentially alter the H/L ratio as a preparatory response to potential injury when inhibiting the flight response, thereby introducing natural variation in stress responses. After breeding, the post-fledging period also requires high energy investments from parents and could lead to trade-offs between parental care, self-maintenance, and survival, resulting in higher stress levels, which is in line with our findings (Stutchbury et al. 2011; Gow und Wiebe 2014). However, other than actual stress, the variation of the H/L ratio in adult birds could also be caused by a natural variation of the H/L ratio in preparation to the challenges of the breeding season.

The lower stress level in juveniles compared to adults may be attributed to their naivety. Specifically, during early development, juvenile birds show decreased responsiveness to stress (e.g. handling stress) and alarm calls and are unable to distinguish between predators and non-predators, which suggests that external stress factors potentially cause less stress in juvenile birds (McLean et al. 1999; Sims und Holberton 2000; Kullberg und Lind 2002; Magrath et al. 2006). Yet, in contrast to our findings and those found in the above-mentioned studies, a study by Jakubas et al. (2011) found that juveniles of reed buntings exhibited higher H/L ratios than adults. Potential explanations for their findings include infection, lack of experience, and inadequate foraging in juvenile birds.

The lower H/L ratio observed in juvenile birds may not, however, be attributed solely to stress. Another factor that could contribute to the lower H/L ratios of juveniles is the ontogeny of their immune system, which is not fully developed at hatching (Quillfeldt et al. 2008; Dehnhard et al. 2011). A study conducted on domestic chickens found a significant increase in the H/L ratio during sexual maturity (Campo und Davila 2002). In another study, thin-billed prions (*Pachyptila belcheri*) showed an increase in the abundance of heterophils with age, while the number of lymphocytes in the blood decreased, resulting in a higher H/L ratio (Quillfeldt et al. 2008). It is plausible that juvenile birds have been less exposed to parasites and pathogens compared to adults, implying that the juvenile immune system may not have been sufficiently stimulated to trigger a reaction and thus result in elevated H/L ratios. In conclusion, the observed higher H/L ratios in adult birds compared to juveniles in our study may be due to several factors, including juvenile naivety, adult stress associated with parental care, different disease histories, and potential influences resulting from the maturation of the immune system and natural seasonal variation of the H/L ratio. These differences in age emphasize the need for age control when evaluating and interpreting stress levels in wildlife.

As we expected, apart from age, also sex of the birds significantly impacted stress levels found in our bird community. Across species, female birds exhibited a significantly higher H/L ratio compared to males. This sex-specific pattern is consistent with other studies showing that females experience higher levels of stress than males during the breeding season (Hõrak et al. 1998; Kilgas et al. 2006). One possible explanation for this is that in addition to incubating and parental care, females have to invest more energy in egg production and breeding efforts than males (Hõrak et al. 1998; Ots et al. 1998; Kilgas et al. 2006; Skwarska 2019). Egg laying

consumes significant resources of female individuals, and elevated levels of reproductive hormones in females may also result in fitness-related costs, which can be reflected in higher stress levels (Bowers et al. 2012). As the sex specific differences in the H/L ratio were also present in juvenile birds, reproduction and parental care cannot fully explain the pattern we found. In rodents a study found larger adrenal glands in females and as a result also significantly higher corticosterone levels than males (Handa und McGivern 2009). In Great Tits, males and females showed differences in their H/L ratio after exposure to social stress with females showing a stronger response than their male conspecifics (van der Meer und van Oers 2015). Furthermore, in a study of rock doves (*Columbia livia*), females exhibited higher genomic responsiveness to stress at all stages of their stress response compared to males (Calisi et al. 2018). This finding may be attributed to hormonal fluctuations associated with their reproductive cycle and parental investment (Calisi et al. 2018). These fixed sex differences may clarify sex-specific variations in stress levels along with breeding investment.

In line with this, we discovered a slightly elevated stress level in birds incubating eggs compared to non-incubating birds, regardless of sex, when considering only potentially incubating birds. Birds with a brood patch displayed a tendency toward higher stress levels than those without a brood patch. This is consistent with the findings of Frigerio et al. (2017) on graylag geese (*Anser anser*), whose study showed that birds that successfully reproduced had higher stress scores than unpaired or broodless birds. The increased H/L ratio observed in breeding birds may indicate a physiological adaptation to breeding, reflecting an investment in nonspecific immune response and changes in endocrinology (Ots et al. 1998; Kilgas et al. 2006; Quillfeldt et al. 2008; Jakubas et al. 2011). However, a study utilizing corticosterone as a stress metric discovered an opposing trend, where males providing greater parental care exhibited lower stress levels than non-incubating females (Edwards et al. 2013). This observation in two shorebird species has been associated with a possible adaptation intended to prevent nest abandonment (Edwards et al. 2013). While individual species may display different patterns, across our forest bird community, female birds hold primary incubation responsibilities, suggesting a significant degree of parental investment and breeding effort. This could partially also explain the elevated stress levels observed in female birds compared to male birds (Hõrak et al. 1998; Ots et al. 1998; Kilgas et al. 2006).

Other than the intrinsic factors, year also had a significant effect on the H/L ratio in our forest bird community, suggesting general variation of the H/L ratio across the years, potentially driven by environmental factors such as temperature and precipitation.

Overall, our study shows that there are general patterns of the effects of sex and age on the H/L ratio across a bird community. Therefore, when applying the H/L ratio as a stress measure it is important to account for this natural variation of the H/L ratio. If and to which extend these factors influence common patterns of stress levels before or after the breeding season remains however uncertain and requires further investigation.

No effect of body condition on the H/L ratio

Contrary to our hypothesis, the H/L ratio in our study was not affected by body condition. This is in contrast to our initial assumption and multiple previous studies. Our initial assumption was that poor body condition would signify limited resource availability and potentially trigger stress, leading to higher H/L ratios which is supported by the results of several previous studies (Gladbach et al. 2010; Plischke et al. 2010; Włodarczyk et al. 2018; Skwarska 2019; Vágási et al. 2020). However, birds in good body condition were found to respond with a greater increase in glucocorticoids, and therefore stress response, when exposed to a stressor (Vágási et al. 2020). One potential explanation for the lack of variation of the H/L ratio in response to the body condition is that birds in superior body condition exhibit a greater variability in their stress response in both directions (Heath und Dufty 1998). As we do not have any knowledge about the presence and intensity of environmental stressors in our study, it is possible that the effect of body condition on stress is masked by the strong stress responses of birds in superior condition after encountering such unknown environmental stressors. This is additionally corroborated by a study that discovered avian specimens in superior body condition having both higher baseline stress levels and a more pronounced stress response (Fokidis 2023). Specifically, among nestlings, those with better body condition tend to have higher H/L ratios compared to conspecific nestlings with poorer body condition (Masello et al. 2009). In this case the higher H/L ratio does not necessarily indicate that nestlings with better body condition were stressed, instead a better body condition might allow nestlings to invest more into their innate immunity (Masello et al. 2009). Other studies have shown that an in-depth analysis of other hematological parameters, such as leukocyte concentration (leukocytes per 10,000 erythrocytes), and the concentration of a specific type of leukocytes

alongside the H/L ratio, might be important to gain a better understanding of the current health status of the bird (Masello et al. 2009). In particular, leukocyte concentrations are closely associated with the overall health of birds, and can reveal further knowledge on the relationship between stress and responses to infections and inflammatory processes (Salvante 2006). While in this study we decided to solely focus on the H/L ratio, using the deep learning approach presented here, these different hematological parameters could be efficiently assessed for bird communities in the wild, which would further advance the application of stress physiology in wild bird communities. Overall, the relationship between body condition and stress seems to be very complex and difficult to interpret in an uncontrolled environment. More pronounced differences in the body condition and a controlled environment could help to gain a better understanding of the effect of body condition on stress.

Phylogenetic signal and stress variability across species

As expected, our findings reveal a significant phylogenetic signal in the H/L ratio data. While the presence of a phylogenetic signal in the H/L ratio data has already been observed across bird species elsewhere (Minias 2019), our study adds to the existing body of knowledge by demonstrating this phylogenetic signal within an entire natural bird community. The ability to respond to stress with physiological adaptations such as the H/L ratio are important to escape dangerous situations and to survive infections (Martin 2009; Martin 2014). The pronounced phylogenetic signal in the stress levels may be attributed to various factors, such as body size, migration and social behavior, which elevate the risk of pathogen infection, necessitating a robust and enhanced immune response (Côté und Poulin 1995; Møller und Erritzøe 1998; Lindström et al. 2004; Minias et al. 2017; Ruhs et al. 2020). Traits influencing immune functions exhibit variation among bird species and have evolved over time (Pap et al. 2015; Minias et al. 2018). Together, these factors contribute to the significant phylogenetic signal observed in our study. The findings underscore the importance of considering phylogenetic relatedness when interpreting H/L ratios across multiple species.

4.6 Conclusions

Overall, we were able to detect general patterns of the H/L ratio in response to intrinsic factors across a forest bird community. These findings underscore the feasibility of conducting an analysis of physiological stress measures across a bird community. By first evaluating common patterns and carefully considering the influence of intrinsic factors and phylogenetic

relationships, this approach may pave the way for analyzing the effect of general environmental or habitat changes on the physiological condition of entire bird communities in future studies. In addition to these noteworthy results on community-level stress, we applied a newly developed deep learning approach to assess stress levels in large bird populations and communities. These results provide crucial insights into how intrinsic factors and phylogeny affect the H/L ratio, emphasizing their role in utilizing this measurement. When combined with the presented neural network approach, the H/L ratio presents itself as a potent and streamlined instrument to assess the physical health of avian populations and communities. As such, it has the potential to serve as a valuable tool for conservation measures.

4.7 Acknowledgements and declarations

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Author contribution

FS contributed to the conception and design of the study, carried out the field and laboratory work, contributed to the conception and development of the neural network approach, performed the statistical analyses, and wrote the manuscript. NF, DS, and SR designed and structured the study and participated in drafting the manuscript. PQ, JM, and YS helped with conceptual and analytical approaches and supported the lab and fieldwork. DS, DG, and SR contributed to fieldwork; YS introduced and helped with both field and laboratory work; MV, MM, NK, HB and BF led the conception and development of the neural network approach, NF, DS, CG and SR contributed to the statistical analysis. All authors contributed to manuscript and read and approved the submitted version.

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

The data used within this article will be made publicly accessible via Dryad once the article has been published. All relevant software and data presented in this article, including the trained neural network models, are openly available at https://data.uni-marburg.de/handle/dataumr/250 (accessed on 27 February 2024). Furthermore, our software repository is publicly available at https://github.com/umr-ds/avibloodcount (accessed on 27 February 2024).

Ethics statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Regional Council of Giessen, Hesse, Germany (protocol code V 54–19 c 20 15 h 01 MR 20/15 Nr. G 10/2019 and date of approval 26 February 2019).

Chapter V: Birds infected with blood parasites react differently to their environment than non-infected birds

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Not published

5.1 Abstract

Climate change is known to be one of the main drivers for biodiversity loss and to accelerate the spread of parasites and pathogens, which in turn can have negative impacts on the population dynamics of their host species. Before populations decline, individuals respond with physiological adaptations to environmental changes and infections. Therefore, measuring the physiological stress response of organisms to their environment has the potential to serve as a valuable warning indicator in conservation. In our study, we aimed to assess how the environmental factors temperature, precipitation, and shrub layer density, as well as infections with blood parasites of the order haemosporida, affected the physiological stress response across a European forest bird community. To achieve this, we captured birds during the breeding seasons of four consecutive years, analysed the prevalence of haemosporidian parasites via DNA sequencing, and related both environmental variables and parasite prevalence to the ratio of heterophils to lymphocytes (H/L ratio) as a physiological stress measure. We found that uninfected birds had lower stress levels at higher temperatures, while infected birds did not show this pattern. We also found a trend indicating that infected birds with higher H/L ratios were more often captured in locations with higher shrub layer densities, whereas uninfected birds tended to have lower H/L ratios at high shrub layer densities. Precipitation had no significant effect on the H/L ratio in the studied forest bird community. Our findings reveal that temperature has the strongest effect on the H/L ratio of birds among the studied environmental factors and that infections with haemosporidian parasites alter the physiological response of birds to their environment.

5.2 Introduction

Changes in environmental conditions, e.g. induced by climate and land-use change, have been identified as one of the main drivers for biodiversity loss and population declines (IPBES 2019). Furthermore, changes in environmental conditions lead to shifts indistribution ranges of parasites, pathogens, which in turn can influence physiological stress measures(Fokidis et al. 2008; Fuller et al. 2012; Wojczulanis-Jakubas et al. 2012; Dunn et al. 2013; Hing et al. 2016; El-Sayed und Kamel 2020). The combination of changing environmental conditions and the higher prevalence of parasites and pathogens can cause population declines in various species (Atkinson und LaPointe 2009; Hing et al. 2016). Such population declines have been widely observed and discussed in recent years (Lafferty und Kuris 1999; Atkinson und LaPointe 2009; Dadam et al. 2019). Before species declines can be observed, changes in environmental conditions and parasite prevalence are known to trigger physiological adaptations in organisms to deal with stress (Young et al. 1989; Wikelski und Cooke 2006; Hing et al. 2016). Yet, the combined effects of environmental changes and parasite infection on physiological stress responses are not fully understood.

Birds are a group of animals in which the physiological response to various environmental factors and its effect on populations has already been studied and discussed previously (Suorsa et al. 2004; Cyr und Romero 2007; Busch und Hayward 2009; Breuner 2011; Bańbura et al. 2013a; Bańbura et al. 2013b; Frigerio et al. 2017; Wingfield et al. 2017). Temperature has a significant impact on bird populations, affecting their survival, reproductive success, and growth rates (Saether et al. 2000; Woodworth et al. 2018; Gardner et al. 2022). This impact is likely mediated by physiological adaptations. The thermoneutral zone, which is the zone where an organisms performs at its optimum, was found to be at 25 to 35 °C in an Australian passerine bird (Lill et al. 2006). When temperatures exceed the thermoneutral zone, birds experience heat stress which can cause changes in blood cell composition, immune response dynamics, and antioxidant status are triggered (Bohler et al. 2021; Weeks et al. 2022; Khan et al. 2023). Other than temperature also other environmental factors, such as precipitation and vegetation structure have been found to affect population dynamics and physiology by triggering a stress response (Schmidt et al. 1992; Huang et al. 2014; Öberg et al. 2015; Brlík et al. 2022). Precipitation has been found to have a positive effect on avian species richness and enhance survival during migration (Liang et al. 2020; Brlík et al. 2022). On the other hand,

rainfall during parental care reduced reproductive success and survival in a passerine bird (Öberg et al. 2015). Vegetation heterogeneity and shrub layer density were positively associated with species richness and affected chick weight in forest ecosystems (Bradbury et al. 2005; Huang et al. 2014; Jakobsson und Lindborg 2017)

Parasite loads, which are partially associated with environmental changes, can also trigger a stress response (Fokidis et al. 2008; Krams et al. 2010; Wojczulanis-Jakubas et al. 2012; Biard et al. 2015). Infections with blood parasites were found to increase the H/L ratio as well as the total leukocyte counts during autumn migration (Wojczulanis-Jakubas et al. 2012). Another study however revealed reduced H/L ratios in an overwintering population of yellowhammers (Dunn et al. 2013). However, other studies did however not find any effect of parasite prevalence on haematological stress parameters (Krams et al. 2010; Dimitrov et al. 2019). As the H/L ratio is however part of the immune system, considering the effects of parasites when analysing such haematological parameters is important (Biard et al. 2015). Due to the costs of infection, parasites and pathogens can also induce changes in population dynamics due to higher resource investment in immunity, which can subsequently affect reproductive effort and success and trigger a physiological stress response (Peach et al. 2008; Cornelius et al. 2014; Plard et al. 2020). Especially in combination with the continually changing environmental factors the impact of parasites and pathogens on population dynamics must not be underestimated (La Martínez-de Puente et al. 2011; van de Crommenacker et al. 2012; Giraudeau et al. 2014; Hing et al. 2016). Therefore, gaining a deeper understanding of the interactive effect of threats such as parasites and environmental stressors on bird populations and communities is crucial.

For understanding and identifying such potential threats to bird populations and communities warning indicators are required (Wikelski und Cooke 2006; Hing et al. 2016; Seebacher et al. 2023). Physiological measures have the potential to serve as such early warning indicators and are frequently used by conservation physiologists (Wikelski und Cooke 2006; Seebacher et al. 2023). Especially physiological stress responses can be a beneficial tool and their application can help to understand changes in habitat selection, altered population dynamics and to elucidate which role parasites and pathogens play in these factors (Wikelski und Cooke 2006; Davis et al. 2008). Chronic stress responses are known to cause trade-offs with other energetically demanding functions such as reproduction and immunity which can ultimately

affect population dynamics (Breuner et al. 2003; Martin 2014). Physiological stress can be measured with numerous methods including the analysis of blood samples. Some physiological measures that can be assessed using blood are circulating stress hormones (Davis und Maney 2018; Maness et al. 2023), haematocrit values (Hõrak et al. 1998; Ots et al. 1998), leukocyte concentration (Davis 2005; Masello et al. 2009) and heterophil to lymphocyte ratios (H/L ratio; Davis et al. 2008; Maness et al. 2023). The H/L ratio is a way to assess long-term stress in birds (Davis et al. 2008). Variation in the H/L ratio is caused by an adaption of the immune system to stressful events or unfavourable environmental conditions. In response to elevated stress hormones lymphocytes are redistributed to lymph nodes, spleen, bone marrow and skin whereas heterophils become more abundant in the blood (Davis et al. 2008). The H/L ratio varies throughout the different life stages of birds and also differs due to intrinsic factors such as sex and age (Hõrak et al. 1998; Strehmann et al. 2024). Some of the stressors triggering changes in the H/L ratios are for example nutritional status (Włodarczyk et al. 2018), parasite infection (Dunn et al. 2013) and habitat composition (Lüdtke et al. 2013; Ribeiro et al. 2022). Due to the high variety of influence factors and when controlling for intrinsic variation, this method has the potential to serve as valuable warning indicator (Davis et al. 2008). It is further of benefit that the requirements and costs for sampling and analysing are comparably low and, with newly developed deep learning approaches for counting avian blood cells, it further does not require much time (Davis et al. 2008; Vogelbacher et al. 2024).

To our knowledge, the combined impact of temperature and parasites on stress levels in birds has not been fully elucidated (Morley und Lewis 2014). Additionally, the utilization of physiological measures to assess the influence of environmental factors and parasites on birds has not yet been evaluated at a community level. In this study we focus on the effect of environmental factors and parasites on the physiological stress response in a European forest bird community. We collected information on the prevalence of haemosporidian blood parasites, which are globally distributed vector borne endoparasites (Valkiūnas 2005) as well as on temperature, precipitation and shrub layer density. Specifically, we investigated whether haemosporidian infection as well as environmental factors had an effect on the H/L ratios of the birds. We incorporated the intrinsic factors sex, age, and body condition into our analysis to account for their influence on the H/L ratio and minimize their potential influence on the outcomes. (Strehmann et al. 2024). Our hypotheses are that bird communities are (1) less stressed at higher temperatures, (2) more stressed during times of high precipitation

levels and (3) less stressed in areas with higher shrub layer densities. Further, we expect that (4) birds with parasites have higher physiological stress levels than uninfected birds and that the effect of parasite infection increases with increasing temperature due to the spring relapse of haemosporidian parasites (Applegate 1971).

5.3 Material and Methods

Field campaigns and Mist-netting setup

Over the course of four field campaigns spanning the breeding seasons from 2019 to 2022, we examined the avian population within a managed forest located in the low-mountain region of Central Germany. During the breeding seasons from mid-March to mid-August each year, we captured birds at ten mist netting sites. At each site we set up 36 meters of mist nets (three times 12 m), with a height of 2.5 meters and a mesh size of 16mm x 16mm. While we only conducted three sampling rounds in 2019, from 2020 onwards we increased the sampling effort to six sampling rounds, resulting in 30 and 60 capture events respectively. During 2019 we only used this standard set up, from 2020 onwards we conducted every second capturing event using high nets measuring five meters in height. We did not use any kind of lure and controlled the nets every 15-20 minutes in order to avoid causing changes in the stress level of the birds. Each individual bird was marked with an aluminium ring for the identification of recaptures and with permission of the Heligoland Bird Observatory (Institute of avian research (IAR), Germany).

Data and sample collection

Of each captured bird we took the standardised measurements wing length, length of the third outermost primary (P8) and body mass [g]. We further determined the sex in all cases where it was morphologically possible as well as the age of the bird. Birds hatched in the respective year were determined as juveniles, birds older than that as adults. Within 30 minutes after the bird flew into the net, we collected a blood sample by puncturing the brachial vein using heparinized capillary tubes. One drop of the thereby collected blood was used to prepare one to two blood smears which were kept dry and dark until further processing in the laboratory. We stored the remaining blood samples on Whatman FTA Classic cards (Whatman, UK) for later DNA analysis, which aimed to determine the sex of the birds and detect infections with haemosporidian parasites. The samples were collected under

permission by the regional council of Giessen, Hesse, Germany (V 54-19 c 20 15 h 01 MR 10/15 Nr. G 10/2019).

Laboratory work

The blood smears were fixated in methanol within 24 hours after the sampling and stained with Giemsa within 21 days after capturing following the standard protocols (Robertson und Maxwell 1990). We then digitized one blood smear of each blood sample using a Leica Aperio AT2 Scanner (Leica Biosystems Nussloch GmbH, Nussloch, Germany) at 40x magnification. The resulting full-resolution scans were then used for training a deep learning approach for automatic cell identification and counting (Vogelbacher et al. 2024). The deep learning approach is based on two deep neural network models. The whole smear scan was cropped into tiles. The first model classified the tiles as suitable for counting or not, the second model is an instance segmentation model which detected and identified the cells in the tiles that were classified as countable (Vogelbacher et al. 2024). By applying the neural network to all our scans, we received counts of all erythrocytes and the leukocytes which were further subdivided in lymphocytes, heterophils and eosinophils. Due to a lack of training data, basophils and monocytes have not been included (Vogelbacher et al. 2024).

The blood from the FTA Cards was used for DNA extraction using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA). For birds of which the sex was morphologically not clearly identified, a PCR was conducted using the P2/P8 primer pair, including both, positive and negative controls (Griffiths et al. 1998). Furthermore, we used the primer pairs HaemNFI/HaemNR3 to detect the presence of Haemosporidian parasites in general, HaemF/HaemR2 to detect *Plasmodium* spp. and *Haemoproteus* spp. and HaemFL/HaemR2L to detect *Leucocytozoon* spp. (Hellgren et al. 2004). Here as well positive and negative controls were included and the parasite lineages found have been uploaded to both, GenBank (OQ311003-OQ311317) and to the MalAvi database (Bensch et al. 2009). The DNA sequences of the mitochondrial cytochrome b gene of haemosporida have been trimmed and assempled using the CodonCode Aligner software (CodonCode Corp., Dedham, MA, USA) and were determined on lineage level using reference sequences from the MalAvi database (Bensch et al. 2009). In the following manuscript we will however only use presence/absence data for haemosporidian parasites and further information on the lineages found can be looked up in the previously published paper by Strehmann et al. (2023).

Environmental data

For assessing vegetation structure, we used Lidar (Light Detection and Ranging) data which was gathered by the Hessian administration for ground management and geoinformation (HVBG) using a Riegl 680i laser scanner. LiDAR data allows to identify differently structured habitat units in landscapes and quantify the variation in vegetation structure within these (Bradbury et al. 2005). The shrub layer density was recorded as the density of non-ground lidar returns in below three meters height and is calculated in percent. The shrub layer density was calculated for every 50 x 50 m raster cell in the forest. Based on this data, we calculated the shrub layer density in a 100 m radius around each mist net location by using the mean value of all grids within the 100m radius around the net. The lowest shrub layer density in this study was 0.29%, the highest 2.4%.

Weather data covering all field seasons was downloaded in hourly resolution from Copernicus Climate Change Service using the ERA5-Land hourly data from 1950 to present (Muñoz 2019). This dataset, which combines model and observational data (Muñoz 2019), served as a comprehensive resource for our study. We utilized the variables 2m above ground temperature (in Kelvin) and total precipitation (in meters) from the dataset, with a resolution of 0.1° x 0.1° grid cells, corresponds to approximately 1.1 km² in the study area, close to Marburg, Hesse. As the H/L ratio is a long-term stress measure, we calculated the mean temperature and precipitation value for the ten days previous to the capturing event for each mist netting site and matched this information to every bird of our dataset. For simplifying the interpretation of the data, we converted the temperature to degrees Celsius (°C) by subtracting 273.15 and the precipitation to millimetre (mm) by multiplying it by 1,000.

Data analysis

All of the statistical analyses were performed using the R software (R Core Team 2023). Prior to data analysis, we excluded recaptured individual birds within a single breeding season identified by their unique ring number, retaining only the initial capture to mitigate potential handling-related effects on the H/L ratio. Recaptures across the years were retained in the dataset (34) as independent measures. Birds of unknown sex, age or reproductive status or with unknown body condition were also removed from the dataset as these factors are known to influence the H/L ratio. The body condition was calculated as an index by calculating the

body condition as weight divided by P8 length for each individual bird and the mean body condition for each species (Johnson et al. 1985). The body condition index was then standardised by multiplying the individual body condition with 100 divided by the mean body condition of all individuals captured within the species. In addition, we only included birds with more than five individuals per family in the analysis and removed birds with low quality blood smears from the dataset of which less than 300 tiles were classified as countable by the first neural network for ensuring good quality of the stress data.

In the final dataset 387 birds of 15 species were included. As there is a phylogenetic signal in the H/L ratio within the forest bird community (Strehmann et al. 2024), we incorporated phylogenetic information in our models. To do so, we downloaded 1,000 phylogenetic trees for all species of our forest bird community from the birdtree website (https://birdtree.org; Rubolini et al. 2015). The tree with the maximum clade credibility was then selected for the further analysis. For fitting phylogenetic generalised linear mixed models (PGLMM; Li et al. 2020) we had to adjust the phylogenetic tree in order to match our individual bird data. Therefore, we added one tip with the phylogenetic signal of zero for every individual of our bird community and linked the tips of the phylogenetic tree to our individual bird data.

The H/L ratio exhibited an approximate exponential distribution. Therefore, for modelling, we applied a log transformation to achieve a normal distribution. To answer our stress related hypotheses, we used the full remaining dataset and fitted a PGLMM testing for the influence of temperature, precipitation, shrub layer density and parasite infection on the H/L ratio. We further included the intrinsic factors sex, age, and body condition index to account for their impact on the variation of the H/L ratio (Strehmann et al. 2024). To account for species-specific differences in the H/L ratio beyond phylogeny due to different habitat preferences, we included species as a random effect. The first model included all possible two-way interactions between the predictor variables. During model fitting, non-significant interactions were iteratively removed, retaining only those that were found to be significant or marginally significant. Specifically, the interactions involving temperature and infection status, as well as shrub layer density and infection status, were retained in the model, while the other predictor variables remained in the model but not as part of any interactions.

5.4 Results

Over the course of four breeding seasons 529 birds of 29 species were captured of which 387 birds of 15 species were included in the dataset of this study. The H/L ratio ranged from 0.004 to 4.626 and the overall mean H/L ratio was 0.268 (±0.328). In total 203 (52.5%) of the birds included in the analysis were infected with haemosporidian parasites (Table 5.1).

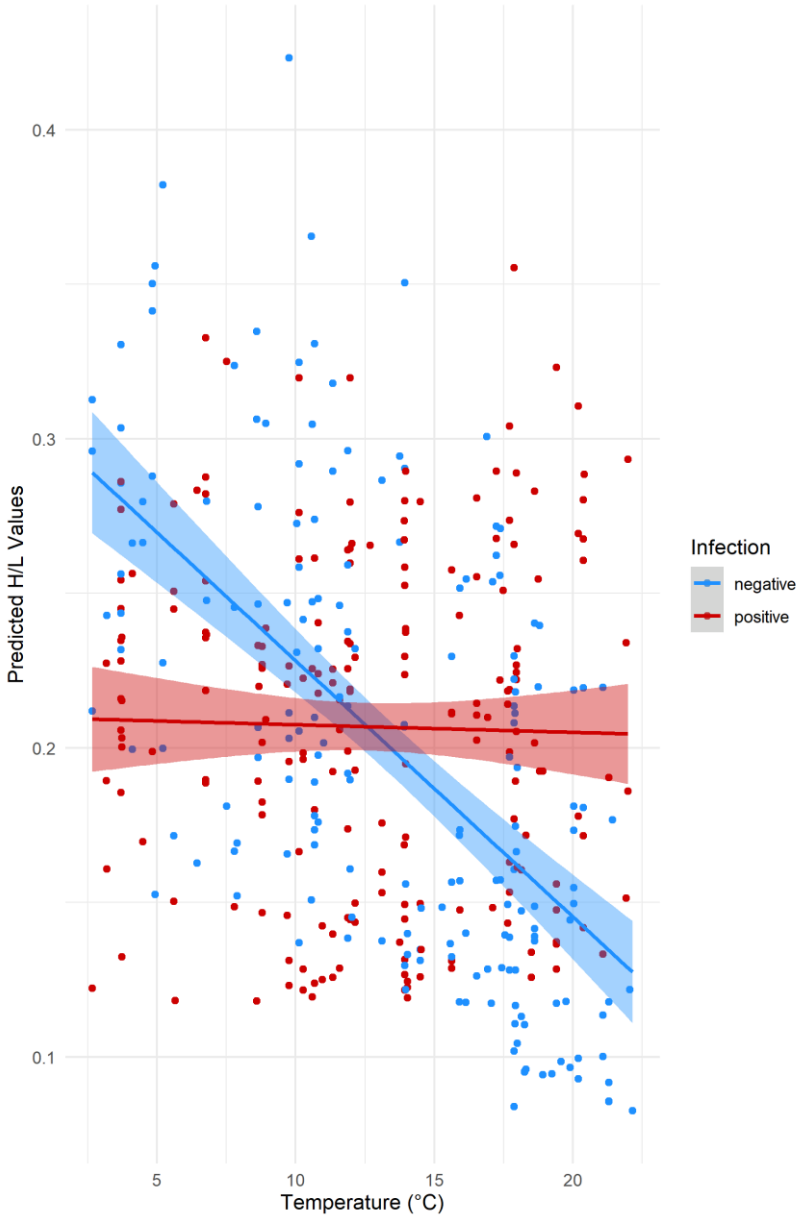

Figure 5.1: Relationship between temperature and predicted H/L ratios in infected and uninfected birds. The scatter points represent individual data points, with colour indicating infection status (red for infected, blue for uninfected). The solid lines represent the fitted regression lines for infected (red) and uninfected (blue) birds, illustrating the trend in H/L ratios with changing temperature.

Table 5.1: Summary of the included sampled bird individuals (N) per species. The number of males (M), females (F), adult birds (Ad) and juvenile birds (Juv) are given. Mean H/L ratio, maximum H/L ratio (H/L max) and minimum H/L ratio (H/L min) of each species are reported. "N infected" shows the number of bird individuals for each species that were infected with haemosporidian blood parasites.

Species	N	M	F	Ad	Juv	Mean H/L	H/L max	H/L min	N infected
Dendrocopos major	11	3	8	8	3	0.569	2.088	0.122	0
Parus major	55	29	26	47	8	0.304	1.106	0.026	49
Poecile palustris	26	10	16	21	5	0.244	0.544	0.004	15
Lophophanes cristatus	5	4	1	3	2	0.244	0.372	0.168	1
Cyanistes caeruleus	24	13	11	23	1	0.443	4.626	0.033	23
Sylvia atricapilla	56	33	23	55	1	0.343	1.663	0.030	29
Phylloscopus sibilatrix	6	3	3	6	0	0.146	0.214	0.112	1
Phylloscopus collybita	11	6	5	10	1	0.215	0.517	0.034	0
Certhia familiaris	15	12	3	11	4	0.204	0.600	0.058	0
Troglodytes troglodytes	13	11	2	7	6	0.394	1.034	0.059	1
Sitta europaea	17	11	6	12	5	0.412	1.941	0.117	10
Turdus philomelos	15	9	6	14	1	0.138	0.329	0.052	14
Turdus merula	36	29	7	36	0	0.163	0.383	0.020	35
Erithacus rubecula	71	48	23	57	14	0.170	0.937	0.026	11
Fringilla coelebs	26	18	8	26	0	0.317	1.193	0.036	14
Σ	387	239	148	336	51	0.277	4.626	0.004	203

The PGLMM model testing the effect of environmental factors and parasites on the H/L ratio revealed that temperature in interaction with infection status had a significant effect on the stress level (Figure 5.1). We further found that infected birds at sites with higher shrub layer densities tended to show higher H/L ratios than uninfected birds at the same location (Table 5.2, Figure 5.2). Precipitation had no effect on the H/L ratio.

Table 5.2: Results of the PGLMM testing the effect of environmental factors and parasites on the H/L ratio.

Fixed effect	Estimate	Standard error	Z-Score	P-Value
Intercept	-0.656	0.052	-12.669	< 0.001 ***
Age (juvenile)	-0.079	0.060	-1.318	0.187
Sex (male)	-0.058	0.037	-1.583	0.114

Body condition index	0.019	0.018	1.097	0.273
Temperature (°C)	-0.071	0.029	-2.489	0.013 *
Precipitation (mm)	0.019	0.018	1.077	0.282
Shrub layer density	-0.031	0.026	-1.171	0.242
Infected (yes)	0.010	0.044	0.219	0.827
Temperature * Infected (yes)	0.088	0.036	2.466	0.014*
Shrub layer density * Infected (yes)	0.061	0.036	1.707	0.088 .

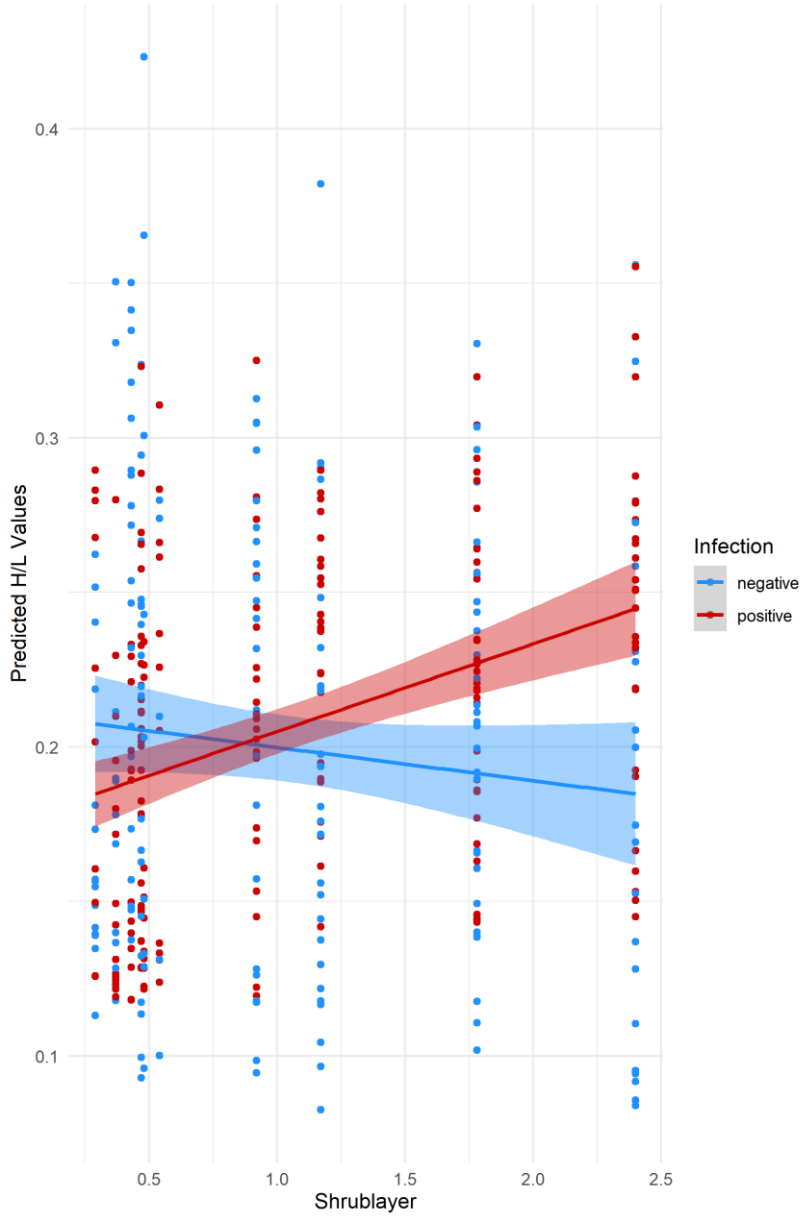

Figure 5.2*:* Association between shrub layer density and predicted H/L ratios in birds, stratified by infection status. The scatter points represent individual data points, coloured according to infection status (red for infected, blue for uninfected). Fitted regression lines for infected (red) and uninfected (blue) birds depict the relationship between shrub layer density and predicted H/L ratios.

5.5 Discussion

We investigated the impact of environmental factors and parasite infection on the H/L ratio as stress indicator. The results showed that infected birds responded differently and less variable with changes in their H/L ratio to changes in temperature than uninfected birds which displayed higher H/L ratios at cold temperatures and lower H/L ratios at high temperatures. Furthermore, we found that infected birds with higher H/L ratios seem to prefer higher shrub layer densities, whereas uninfected birds tend to be less stressed at high shrub layer densities.

Of the environmental factors assessed in this study, only temperature in interaction with parasite infection had a significant effect on stress. In uninfected birds, as expected, the H/L ratio was lower at higher temperatures, indicating lower physiological stress in the birds. As the thermoneutral zone in passerine birds is at 25 to 35°C, this was to be expected. In their thermoneutral zone birds have to invest less investment in thermoregulation when compared to colder temperatures and at warm temperature the food availability is better due to higher insect activity (Brodin et al. 2017; Watson et al. 2023). Below or above their thermoneutral zone, birds experience stress which results in elevated H/L ratios (Xie et al. 2017). While the temperatures in our study system did not exceed the thermoneutral zone of the birds, with ongoing global warming heat stress may become a more important driver on population dynamics in future.

The absence of variation in the H/L ratio among infected birds in response to temperature changes, to our knowledge, has not been previously observed. It is possible that due to the parasite infection, infected birds may lack the capacity to adapt their H/L ratio in response to temperature, as their immune system is preoccupied with combating the infection and may be less flexible in responding to stress. Further investigation is needed to better comprehend this lack of reaction. Exploring other haematological parameters, such as the concentration of leukocytes per 10,000 erythrocytes and the level of parasitaemia, could provide valuable insights into this phenomenon (Schoenle et al. 2017). Furthermore, with rising temperatures in spring, typically a spring relapse of malaria infection occurs which could mask the positive effect of warming temperatures on the H/L ratio (Applegate 1971; Cornelius et al. 2014).

Other than with temperature, parasite infection in interaction with shrub layer density had marginal effects on the stress response in birds. We found that infected birds captured at high shrub layer densities had higher H/L ratios than at low shrub layer densities or uninfected

birds at high shrub layer densities. As the shrub layer density is a factor the bird can choose and is not exposed to, this pattern is likely driven by behavioural adaptations of the birds. It is possible that infected birds prefer habitats with higher shrub layer densities for shelter and better protection of predators. Studies on such changes in habitat preferences and behaviours of infected birds have however to our knowledge not been carried out so far. Another possible explanation is based on the lower activity of infected birds (Mukhin et al. 2016). If a bird suffers from severe blood parasite infection it might not move as much as an uninfected bird (Mukhin et al. 2016). In areas with higher shrub layer densities there are more perching possibilities and therefore flight distances are potentially shorter. Thus, infected birds could prefer this habitat type and were captured on their short distance flights.

In contrast to temperature and shrub layer density, precipitation had no effect on the H/L ratio. Previous studies found that This may however be different in other habitats such as open landscapes as on the side the canopy cover protects animals from direct rainfall and on the other side forest maintain a more humid microclimate due to the shading and canopy closure (Arx et al. 2012). So even though our study did not find an effect of precipitation on the stress levels of a forest bird community, in other habitats such an effect might be present. This remains however to be confirmed by future studies.

5.6 Conclusions

Overall, we found that we expected, temperature significantly impacts the stress levels of forest birds with uninfected birds being less stressed at warmer temperatures. To our surprise we also discovered that birds infected with blood parasites respond differently to their environment than uninfected birds and do not vary their H/L ratio in response to temperature and seem to prefer different higher shrub layer densities when stressed. Future studies should focus on how an infection with haemosporidian parasites alters the stress response and interactions of birds with their environment using either an experimental approach or a tracking system which allows to evaluate the activity of the birds. It might furthermore be interesting to test whether the level of parasitaemia determines the variability of the H/L ratio of birds in response to their environments (Schoenle et al. 2017). Monitoring the breeding success of the sampled birds and measuring additional stress indices such as corticosterone or heat-shock proteins (Ibáñez-Álamo et al. 2020) could further help to gain a better understanding of the fitness consequences of a changing environment and parasite infection

This would then help to further evaluate physiological stress measures as warning indicator for conservation.

5.7 Acknowledgements and declarations

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Author contribution

FS contributed to the conception and design of the study, carried out the field and laboratory work, performed the statistical analyses, and wrote the manuscript. NF, DS, and SR designed and structured the study. PQ, JM, and YS helped with conceptual and analytical approaches and supported the lab and fieldwork. DS, and SR contributed to fieldwork. YS introduced and helped with both field and laboratory work. NF, DS, and SR contributed to the statistical analysis. All authors contributed to manuscript revision and have read and approved the submitted version.

Ethics statement

The animal study protocol was approved by the Institutional Review Board (or Ethics Committee) of Regional Council of Giessen, Hesse, Germany (protocol code V 54–19 c 20 15 h 01 MR 20/15 Nr. G 10/2019 and date of approval 26 February 2019).

Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

The data used within this article will be made publicly accessible via Dryad repository

Chapter VI: Synthesis

The drivers of the biodiversity crisis and the spread of parasites are significant threats to wildlife populations (Daszak et al. 2000; Grazer und Martin 2012; Cunningham et al. 2017; IPBES 2019; Jaureguiberry et al. 2022). Despite extensive research, the diversity and interactions and consequences for the hosts of endoparasites, particularly avian malaria and other haemosporidians, are still not fully understood (Valkiūnas 2005; Fuller et al. 2012; Dunn et al. 2023). These parasites not only decrease host survival but also reduce reproductive success, which are crucial factors for population dynamics (van Riper et al. 1986; Atkinson und van Riper 1991; Bennett et al. 1993; Bueno et al. 2010; Knowles et al. 2010). Furthermore, stress can have a detrimental impact on the fitness of birds, highlighting the importance of understanding physiological adaptations to environmental change (Moberg 2000; Romero 2004; Wikelski und Cooke 2006; Cyr und Romero 2007; Davis et al. 2008; Martin 2009; Davis und Maney 2018; Seebacher et al. 2023). Measures such as the H/L ratio in birds provide insights into physiological responses to stress in individual species and enable the identification of potential threats (Davis et al. 2008; Krams et al. 2011; Sheriff et al. 2011; Bańbura et al. 2013a; Dunn et al. 2013; Frigerio et al. 2017; Bain et al. 2019; Ibáñez-Álamo et al. 2020; Ribeiro et al. 2022). However, their application across entire bird communities is still unexplored. In this thesis I investigate the diversity and prevalence of endoparasites and evaluate and improve the utility of the H/L ratio as a stress assessment tool across a forest bird community.

6.1 Unveiling haemosporidian parasite dynamics: High prevalence and diverse susceptibilities in a European forest bird community

Even though parasites have long been underrepresented in ecological and conservation studies, more and more studies are showing the importance of understanding the impact of parasites on the ecosystem (Gómez et al. 2012; Gómez und Nichols 2013; Okamura et al. 2018). Parasites play a significant role in maintaining ecosystem balance and regulating population dynamics through effects on host species' abundance, behavior, and fitness (Gómez und Nichols 2013; Poulin 2014). However, driven by climate change, parasites and

their vectors are shifting their distribution range and get in touch with new host species (Daszak et al. 2000; Jones et al. 2008; Fuller et al. 2012; Cunningham et al. 2017). Due to the lack of co-evolution and depending on the host specificity of the parasite, this can cause a significant threat to the novel hosts (van Riper et al. 1986; Atkinson und van Riper 1991; Atkinson und LaPointe 2009; Fuller et al. 2012). The current distribution ranges of endoparasites and the current host-parasite interactions are however not fully understood (Fuller et al. 2012; Dunn et al. 2023). Therefore, such range- and host shifts might go unrecognized.

In Chapter II, I investigated the endoparasites of a European forest bird community, focusing on *T. gallinae* and parasites of the order Haemosporida, to enhance our understanding of their prevalence, diversity, and underlying host-parasite networks in a managed European forest. I collected samples from 483 birds and employed DNA sequencing for parasite detection. Utilizing the results, I constructed a bipartite network and calculated network parameters to gain insight into the host-parasite network. Additionally, I examined parasite prevalence and diversity differences among avian families and assessed the impact of parasite diversity on prevalence. While no *T. gallinae* infections were detected, about half of the community (48.1%) was infested with blood parasites. However, parasite infections were not uniformly distributed across the avian community, with significant variations in prevalence and diversity observed among species. Turdidae and Paridae were common hosts for blood parasites, whereas no infections were found in Picidae or Certhiidae. Notably, parasite prevalence did not predict parasite diversity. Compared to other European studies I further found higher prevalences of *Haemoproteus* spp. and *Leucocytozoon* spp. while *Plasmodium* spp. was comparably rare in my study. All of parasite lineages have been previously described; I did however discover novel host-parasite interactions in my study system. My findings show that certain bird species and families, particularly common species, are more susceptible to blood parasites than others and suggest regional differences of infection patterns.

6.2 Advancing avian haematology: A deep learning tool for automated cell counting in blood smears

In recent years, researchers have increasingly focused on understanding the physiological adaptations of animals to their environment for the purposes of nature and species

conservation (Wikelski und Cooke 2006; Davis et al. 2008; Davis und Maney 2018; Seebacher et al. 2023). This has led to the formation of the discipline of conservation physiology (Wikelski und Cooke 2006; Dantzer et al. 2014; Seebacher et al. 2023). One possibility to gain an understanding of how animals react to their environment and to identify possible threats, is by measuring and analysing the stress response (Wikelski und Cooke 2006; Sheriff et al. 2011; Dantzer et al. 2014; Sorenson et al. 2017; Seebacher et al. 2023). In birds there are plenty options available, with a frequently used and promising one being the analysis of avian blood cells from blood smears. Especially the ratio of heterophils to lymphocytes (H/L ratio) is known to vary with stress (Campo und Davila 2002; Davis et al. 2008). So far, the evaluation and cell counting of the blood smears had to be done manually, which is on the one hand a very time-consuming and tedious task and on the other hand also requires intensive training and is prone to observer bias (Ruiz et al. 2002). Furthermore, only small parts of the blood smear can be assessed which is another potential cause for inaccurate results.

To increase the accuracy and improve the effectiveness of this method, in Chapter III, I collaborated with computational scientists for developing a deep learning approach for automatic identification and counting of avian blood cells from digitized blood smears. Our approach consists of two neural network models: one selects regions of the blood smear with high quality and mono-cell layer, which are therefore suitable for further analysis, and a second one to detect and identify the cells within these regions. Both models achieve each over 90% precision. The high accuracy of our deep learning approach and the larger area of the blood smear analysed offer a simplified yet precise method for analysing avian blood smears. By automating the process of cell identification and counting, our approach significantly reduces the time and labour required for analysis while minimizing the potential for observer bias. Additionally, our method allows for the efficient analysis of large sample sizes, making it particularly advantageous for ecological studies where manual evaluation is not feasible. Overall, our approach enhances the usability of the H/L ratio in ecological research, increasing its attractiveness as a tool for ecologists and conservation physiologists. In addition, our approach can be expanded with more training data to analyse blood samples from other bird orders, as well as other vertebrates, such as reptiles, amphibians, or fish. It would also be possible to analyse blood parasites with additional training data.

6.3 The role of age, sex, and reproduction in shaping H/L ratio variability in a forest bird community

While the H/L ratio is commonly employed for stress assessments in birds, interpreting the results can be challenging (Hõrak et al. 1998; Campo und Davila 2002; Davis et al. 2008; Quillfeldt et al. 2008; Masello et al. 2009; Müller et al. 2011; Davis und Maney 2018). This difficulty arises from natural fluctuations in the H/L ratio influenced by intrinsic factors such as sex, age, and reproductive status, leading to a lack of baseline stress levels (Campbell und Dein 1984; Tablado und Jenni 2017; Skwarska et al. 2019). Furthermore, thus far, the H/L ratio has not been used to assess stress patterns across bird communities, but rather focused on single host species and fluctuations within and across populations (Kilgas et al. 2006; Krams et al. 2010; Bańbura et al. 2013a; Bańbura et al. 2013b; Ibáñez-Álamo et al. 2020; Ribeiro et al. 2022). In order to broaden the applicability of the H/L ratio to stress patterns in bird communities, it is important to understand how phylogenetic relatedness and intrinsic factors such as age, sex, body condition and reproductive status affect natural patterns of the H/L ratio within the chosen population or community.

In chapter IV I therefore investigated the natural differences of the H/L ratio during the breeding season caused by sex, age, body condition and breeding condition. I discovered a significant phylogenetic signal of the H/L ratio across the bird community, indicating the shared evolutionary background of closely related species. Taking this into account, I utilised phylogenetic generalised linear mixed models (pglmm) for analysing the impact of sex, age and reproduction on the H/L ratio. The results showed that both sex and age have a significant influence on the H/L ratio. Female birds had higher H/L ratios than males and adults have higher H/L ratios than juveniles. The results of the reproductive status were not as strong, but I discovered that birds with a brood patch and therefore involved in actively incubating the eggs, tend to have higher H/L ratios. In contrast to my expectations, body condition did not affect the H/L ratio. These results do not necessarily reflect a stress response but rather a physiological adaptation of the immune system to the current phase of life and the ontogeny of the immune system in juvenile birds. In this study I found common patterns of how intrinsic factors influence the H/L ratio within a forest bird community. These findings highlight the significance of considering both phylogeny and intrinsic factors when analysing the

physiological response of birds at the community level, particularly in the absence of available baseline levels.

6.4 Haemosporidian parasite infection causes altered physiological responses to environmental changes

Changes of environmental conditions naturally induce physiological adaptations in any organisms (Wikelski und Cooke 2006; Seebacher et al. 2023). This is the case for both natural fluctuations, as for example due to seasonal changes but also for unpredictable extreme events induced by both abiotic and biotic factors (Romero 2002, 2004; Fokidis et al. 2008; Norte et al. 2009; Jakubas et al. 2011; Dunn et al. 2013; Strasser und Heath 2013; Nwaogu et al. 2019; Bohler et al. 2021; Ribeiro et al. 2022). The ongoing climate change can however influence both the seasonal dynamics and the frequency of extreme climate events (Delmonaco et al. 1999; Ma et al. 2015; Moore et al. 2015). Rapid and unforeseen changes in the environment of organisms usually trigger a stress response (Sheriff et al. 2011; Martin 2014; O'Dell et al. 2014). Prolonged stress has however been found to diminish the reproductive success and can therefore in the long term negatively affect the population dynamics (Hõrak et al. 1998; Ilmonen et al. 2000; Breuner 2011; Edwards et al. 2013; Strasser und Heath 2013; O'Dell et al. 2014).

In chapter V of my thesis, I analysed the impact of the abiotic factors temperature and precipitation, and the biotic factors shrub layer density and parasite prevalence on the H/L ratio of birds in a forest bird community. By doing so, I aimed to detect general patterns of stress response of forest birds. I expected birds to be less stressed at higher temperatures and higher shrub layer densities and more stressed in periods with high precipitation and when infected with parasites. As expected, increased temperatures caused significant lower H/L ratios across uninfected birds within the community. Infected birds however did not vary their H/L ratio in response to temperature. This lack of variation could have been caused by a lack of capacity for adaptation. Heterophils and lymphocytes are both part of the immune system of birds, during an infection, the activity of the immune system could reduce the ability to respond to environmental change with an altered H/L ratio. Furthermore, infected birds tended to have higher stress levels at sites with higher shrub layer density, while uninfected birds, as expected, tended to be less stressed at high shrub layer densities. Other than

temperature, birds can choose to be at sites with higher or lower shrub layer density. Therefore, this pattern could be due to behavioural adaptations of the birds. It is possible that stressed birds suffering from haemosporidian infection downregulate their flight activity to save energy. At high shrub layer densities, the flight distances are potentially shorter which is why stressed infected birds might prefer this habitat. Precipitation had no effect on the H/L ratio at all during our sampling period. Overall, I found a significant effect of temperature on the H/L ratio in general, however more interestingly, I discovered that birds infected with haemosporidian parasites respond differently to their environment.

6.5 General conclusions

The results of this thesis show that both environmental factors as well as infection affect the physiological responses of birds. I found that almost half of all forest birds were infected with blood parasites, discovered that intrinsic traits such as sex and age cause significant alterations in the H/L ratio of the birds and that birds infected with haemosporidian parasites display a different stress response and potentially alter their behaviour. This leads me to the following major implications:

Firstly, blood parasites are highly prevalent in the by us studied managed European forest and infect a large variety of host species. Although haemosporidian parasites have co-evolved with their avian hosts, other studies found that they can lower the reproductive success of birds (Valkiūnas 2005; Knowles et al. 2010; Clark et al. 2014; Lauron et al. 2015; Clark und Clegg 2017; Ellis et al. 2020). Nevertheless, currently they do not seem to pose a major threat to bird populations. The families with the highest parasite prevalence among avian species were Turdidae and Paridae. These families are generally common and many of their species are frequently observed in various habitats, making them rather generalist (Bezzel 1993). However, due to the high abundance of the birds and other threats such as land use change, predators and other pathogens, effects of blood parasites on their population dynamics may go unnoticed. In future however, climate change is expected to increase the risk of haemosporidian infection even further and thereby also the impact on host populations (Fuller et al. 2012; Clark et al. 2014; Bodawatta et al. 2020). Furthermore, the shift in distribution ranges of parasites and their vectors could result in parasite lineages infecting novel host species (Fuller et al. 2012; Clark et al. 2014). While transmission to new hosts could

be a dead end for some host-specialist parasites, generalist parasites may successfully infect new host species (Valkiūnas 2005; Clark und Clegg 2017; Ellis et al. 2020). Due to the lack of adaptation, this could cause more severe cases of infection and unpredicted negative population dynamics of the hosts (Atkinson et al. 2000; Atkinson und LaPointe 2009; Clark et al. 2014; Samuel et al. 2015; Names et al. 2021). In island bird populations, the introduction of blood parasites has led to severe population declines and even extinction of endemic Hawaiian species before adaptation took place (van Riper et al. 1986; Atkinson et al. 2000; Atkinson und LaPointe 2009; Samuel et al. 2015). The negative impact of parasites on host populations are not necessarily due to reduced survival rates, but also a reduction of reproductive effort or success can on the long-term cause population declines (Knowles et al. 2009; Cornelius et al. 2014; Romano et al. 2019). Especially birds already dealing with infection of blood parasites might not have the immunological and physiological capacity to deal with another, novel parasite and pathogens (Agliani et al. 2023). The negative effect of such novel infections may therefore be stronger on birds that are already preloaded with other parasites (Agliani et al. 2023). These results demonstrate the significance of monitoring parasites and pathogens in wild birds to identify new parasites early and take appropriate measures to limit their spread.

Moreover, by evaluating the H/L ratio of birds at a community level, I detected general patterns of intrinsic fluctuations in the H/L ratio driven by intrinsic traits. In combination with the significant phylogenetic signal, these results contribute to a better understanding of whether and to what extent a physiological stress response can be applied across species in an avian community. This helps to discover general patterns and make general statements. For the H/L ratio no baseline stress levels are available as it does not only represent the stress response but is also influenced by other factors such as infections and resource availably (Vleck et al. 2000; Davis et al. 2008). Therefore, when evaluating the H/L ratio as a stress response, the results can only be related to other individuals within the respective ecosystem, population, community or experimental setup. It is important to consider the intrinsic variation in the data based on phylogeny, sex, and age and to correct for these factors in model when evaluating the effect of stressors on birds both in on population level, but also on community level (Campo und Davila 2002; Kilgas et al. 2006; Quillfeldt et al. 2008; Jakubas et al. 2011; Minias 2019). The influence of sex and age on the H/L ratio and potentially also other stress measures however also depends on the season (Romero 2002; Jakubas et al. 2011;

Dunn et al. 2013). The challenges and requirements of birds differ depending on the season. During spring, their primary focus is on finding a suitable partner and territory, building a nest, and laying and incubating eggs. Later, the focus shifts to feeding and raising the chicks. In the case of migratory birds, migration poses another challenge that is not sex-dependent, unlike reproductive challenges. Therefore, the effects of intrinsic traits on the chosen stress measure must be evaluated depending on the season or experimental setup to account for such natural variation in the stress data.

In the last chapter of my thesis, I evaluated the impact of temperature, precipitation, shrub layer density, and parasite prevalence on the H/L ratio of birds within the studied forest bird community. The analysis presented here, revealed notable patterns, particularly concerning temperature. Despite finding no direct effect of parasite infection on the H/L ratio of birds in my study, their responses to environmental factors varied. Infected birds appeared less adaptable in adjusting their H/L ratio to changes in temperature. Furthermore, birds with higher stress levels seemed to favour habitats with denser shrub layers. These findings prompt further interpretation. Given that the H/L ratio is part of the immune system, it is plausible that infected birds may lack the capacity for variation in their H/L ratio due to immune system engagement in combating infection (Gross und Siegel 1983; Davis et al. 2008). To better understand the lack of change in the H/L ratio among uninfected birds, additional data such as levels of parasitaemia and further stress metrics would be necessary. Parasites may not only influence H/L ratio variability in response to environmental changes but also affect other stress responses like corticosterone, heat stress, or oxidative stress (O'Dwyer et al. 2020; Jiménez-Peñuela et al. 2023). A better knowledge of how parasites affect the physiology of their hosts and the ability to adapt to the environment will further help to understand and evaluate the fitness costs of infection. Such insights can then allow to perform more in-depth assessments of the risks for population declines, shifts in community structures and (local) extinctions.

In conclusion, it is crucial to monitor parasites and pathogens in bird communities due to their potential to trigger range shifts in response to climate change and their capacity to alter bird populations and communities. Additionally, certain physiological measures, such as the H/L ratio, hold promise in providing insights into general stress patterns across communities. However, it is essential to acknowledge the limitations of analysing general stress patterns

across bird communities, particularly when the predictor variable is not independent of species-specific preferences. While the H/L ratio can be valuable for assessing the impact of weather conditions, climate change, and habitat modifications on bird communities, its application has constraints. For example, when assessing conservation measures for particular target species, applying physiological responses across bird communities for evaluation may not be effective. Certain forest species, such as grouse species (Tetraonini), require forest patches with diverse structures and coniferous trees as habitats (Storch 2002; Rösner et al. 2012). Targeted conservation efforts for these species aim on diversification of habitats and while this is beneficial for the target species, non-target species may not respond or even negatively respond to such habitat changes. The habitat preferences of black woodpeckers for example differ greatly from those of grouse. Black woodpeckers prefer beech forests and need old growth trees for building cavities (Rosa et al. 2016). Therefore, when evaluating the impact of conservation efforts on physiological stress responses, it is important to analyse only the target species. Non-target species may exhibit opposite responses, which could mask the positive effects of the measure on the target species. The same applies to the installation of nesting boxes. While cavity-nesting species benefit from the offer, such conservation measure has no positive effect on ground-nesting species. In such cases, a community-based analysis of the physiological stress response has no advantages.

Physiological stress measures can provide valuable insights into the overall stress responses of bird communities to environmental changes affecting all species in a similar way such as loud noise, higher predator abundance, human disturbance or changes in temperature or precipitation. However, when designing research studies, it is important to consider the life strategies, habitat preferences, and other species-specific characteristics that could affect birds in the chosen study system. The use of stress measures should be based on the expectation of a consistent response across all species included. Alternatively, if variations in stress responses are anticipated, it is essential to supplement general assessments with species-specific evaluations to ensure the accuracy and relevance of the findings. Using physiological measures to assess the well-being of local populations and entire communities, as well as to evaluate conservation efforts, offers great potential for the immediate detection of both positive adaptations, such as increased resilience to environmental stressors, and negative adaptations, such as compromised reproductive success or immune function.

Although my doctoral thesis focussed exclusively on the physiological stress response of birds, the approaches I applied are not limited to birds and can also used to study other vertebrate communities (Davis et al. 2008). The applicability of the H/L ratio (in birds and reptiles) or N/L ratio (neutrophil to lymphocyte ratio, in amphibians, fish and mammals) has already been confirmed in many studies (Harris und Bird 2000; Wojtaszek et al. 2002; Bilandzić et al. 2006; Obernier und Baldwin 2006; Chen et al. 2007; Davis et al. 2008; Davis und Maerz 2008). As in birds, the erythrocytes of most vertebrates (with the exception of mammals) have a nucleus (Claver und Quaglia 2009). This means that the deep learning approach developed by us, which identifies erythrocytes based on the nuclei, can also be adapted to other vertebrate groups. However, as with birds, the influence of intrinsic factors and phylogeny must be investigated before analysing the physiological stress response across the community in order to correct for this. In invertebrates, the stress measures used in this study cannot be used, but hormone and neurotransmitter levels can be used to assess physiological stress and identify stressors in invertebrates.

Furthermore, the influence of parasites and pathogens should not only be investigated in birds, but also in other taxa. While parasites and pathogens in birds are relatively well studied due to their annual migration and their importance for emerging infectious diseases (EIDs), other taxa are not as well studied (Reed et al. 2003; Fuller et al. 2012). Yet the potential effects of emerging infectious diseases have been observed in other taxa, such as stone crayfish and fire salamanders (Lastra González et al. 2019; Jussila et al. 2021).

In summary, continued research into physiological stress responses across taxa is essential for comprehensively understanding population dynamics and the biodiversity crisis, emphasizing the need for holistic approaches in conservation efforts.

Chapter VII: Research perspectives

In my thesis, I have analysed endoparasite diversity and explored the use of the H/L ratio as a physiological stress indicator across a bird community. While my research has provided valuable insights about general patterns of the H/L ratio and the applicability of this physiological measure across a community, it has also raised intriguing research questions. In this final chapter, I will introduce some of these questions, discuss their significance, and propose potential study designs for future research.

7.1 Exploring Avian Parasites and Pathogens: Enhancing Monitoring and Understanding Disease Dynamics

During the analysis of the host-parasite network and when comparing it to the MalAvi database (Bensch et al. 2009), I detected novel host-parasite interactions and other recent studies even detected unknown parasite lineages. The remaining knowledge gaps regarding the diversity, distribution and interactions of blood parasites limit the ability to recognise changes at an early stage. To address these gaps, large-scale monitoring covering different habitat types and host species could be instrumental for determining current distribution limits and for learning more about established host-parasite interactions as well as on the long-term to detect changes in the host-parasite community. For example, in Germany, the "Integrated Monitoring of Songbird Populations" (IMS) project, initiated by the German bird observatories and the Dachverband Deutscher Avifaunisten (DDA) since 1999, aims to collect data on population dynamics, reproduction rates, and survival rates at a state-wide or nationwide level (Bairlein et al. 2000). Incorporating random sampling for haemosporidian blood parasites within the framework of the IMS project could provide valuable data for central evaluation and analysis. However, implementing such a project faces challenges, particularly as many bird ringers involved in IMS are volunteers without access to animal testing permissions required for blood sample collection. Nevertheless, with careful planning and effort, this project could significantly enhance our understanding of host-parasite interactions, the distribution limits of parasites, and their prevalence across different regions and habitats. One idea for carrying out such a project would be to have one or more scientists with animal testing permits visit the various IMS projects for one day and collect random samples. Alternatively, as some IMS sites are also operated by scientists, it would be possible

to include only these sites in the study for the time being. The responsible bird ringers will take random blood samples from the captured birds. These samples are then collected and analysed. Through targeted training, the number of bird ringers with animal experimentation permits could be gradually increased and the project developed into a sustainable long-term project.

To deepen our understanding of haemosporidian parasite infection dynamics, it is crucial to incorporate vectors into our analysis. Mangy European bird species migrate during winter, leading to uncertainty about the transmission location. Identifying potent vectors of blood parasites is challenging. PCR-based methods alone may detect parasites in insects but often yield positive results based on previous blood meals, without necessarily identifying potent vectors (Bernotienė et al. 2019). Combining PCR-based analysis with microscopy allows for the identification of vector individuals carrying parasite DNA and the search for sporozoites (the infective stage of the parasite) to confirm their potency (Bernotienė et al. 2019). Despite the method's precision requirements and the need for extensive knowledge of Haemosporida developmental stages, including vectors in blood parasite research enhances our understanding of infection dynamics. It helps identify transmission regions for different parasite lineages and informs risk assessments regarding potential range expansions of haemosporidian parasites. Investigating haemosporidian parasites in both vectors and hosts within a single ecosystem can help to disentangle infection pathways, leading to a better understanding of infection dynamics. This, in turn, can facilitate the establishment of appropriate conservation measures if necessary. For human malaria measures to decrease risk of transmission are already in place. Malaria prevention projects aim on reducing vector populations by for example capturing adults with mosquito traps and odor baits in combination with larval source management (Knols et al. 2023). The recently developed precision guided sterile insect technique is another successful method to reduce vector populations (Smidler et al. 2023). In this method mosquitos are being genetically modified for sterilization (Smidler et al. 2023). Crossing experiments showed that females mated to modified males were complete or nearly complete sterile which on the long term leads to a significant reduction of malaria transmitting vectors (Smidler et al. 2023). It is currently unclear whether or not methods similar to those discussed will be employed in the future to control blood parasites in birds, and if so, to what extent this would be ecologically justifiable.

However, if blood parasites become a greater threat in the future than they are currently, there is the possibility of employing such methods.

While haemosporidian parasites currently receive significant attention from researchers (Dunn et al. 2023), it is important to recognize that there are numerous other parasites and pathogens posing potential threats to bird communities. Assessing a broader range of parasites and pathogens within a community provides valuable insights into their influence on community structure, population dynamics, and the ability for physiological adaptations to environmental changes at the individual level. For instance, in *Turdus merula*, Agliani et al. (2023) observed that the severity of lesions was heightened in birds infected with both *Plasmodium* spp. and the Usutu virus when compared to individuals with single infection. Focusing solely on one type of parasite or pathogen may therefore overlook interactive effects among different pathogenic agents, highlighting the need for a comprehensive approach to studying avian health and disease dynamics. While haemosporidian blood parasites solely infect birds, other diseases such as the West Nile Virus can switch hosts and also infect livestock and humans (Michel et al. 2019). Adopting the One Health approach (FAO et al. 2008; Gruetzmacher et al. 2021), a more comprehensive monitoring of diseases in bird communities can aid in understanding disease dynamics and the effects of multiple infections, ultimately enabling better risk assessment for emerging diseases.

7.2 Physiological Stress in Bird Communities: Implications for Population Dynamics and Conservation Opportunities

In my thesis, while exploring physiological stress responses and parasites, I referred to previous studies that analysed how these factors can reduce reproductive success in birds (Hõrak et al. 1998; Ots et al. 1998; Krams et al. 2010; Breuner 2011; Romano et al. 2019). To gain a deeper understanding of the impact of stress and parasite infestation on population dynamics, future studies could focus on analysing the reproductive success of the studied bird individuals. Specifically, investigating the combined effect of an elevated H/L ratio and parasite infection, which, to my knowledge, has not been thoroughly analysed before, could provide valuable insights. However, implementing such studies in bird communities, especially those comprising numerous species, poses challenges due to limited access to nests, which are essential for evaluating reproductive success. Although it is possible to access nests of certain species, such as wood warblers, which are ground breeders, monitoring reproductive

success of other species is less feasible due to limited access to nests. The installation of nest boxes and the monitoring of the reproductive success of the birds nesting in them can be considered as a viable solution, but it is important to note that this method can only be applied to a part of the bird community, as not all bird species use nest boxes. Conducting a study on the impact of stress levels and parasites on reproductive success would provide valuable insights and clarify their influence on population dynamics.

Another research perspective, based on the findings from my thesis, is to analyse the behaviour of stressed and infected birds. In chapter V, I discussed the results that infected birds with relatively high H/L ratios appeared to favour habitat structures with a higher shrub layer density. One theory to explain this result was that stressed and parasitized birds may have a reduced flight activity and therefore prefer denser habitats with more perching opportunities. To test this hypothesis, conducting a behavioural study of birds with known stress levels and infection status would be beneficial. However, such a behavioural study of wild birds is often not feasible, especially in dense habitats such as forests. Nevertheless, technical approaches, such as GPS transmitters or the use of VHF telemetry to classify the activity of the birds, could make such a research project possible (Gottwald et al. 2023). By applying these methods, information on the birds' activity is available for every second over a period of a few weeks. This enables testing whether infected birds are genuinely less active than uninfected birds and whether the H/L ratio also affects activity patterns.

The use of physiological measures at a community level offers further opportunities to comprehend the impact of environmental factors on bird communities. Additionally, various physiological measures, each providing insights into different aspects such as stress, immunity, and nutritional status, enhance our understanding beyond the H/L ratio. However, their applicability across communities and species requires thorough evaluation. Physiological measures can aid in comprehending the effects of environmental changes on physiological adaptations within a community, as well as assessing habitat quality and habitat-related stressors across similar communities. The use of physiological measures within and across communities raises additional research questions, which I will discuss in more detail below.

(1) An intriguing avenue for future research is to evaluate the influence of environmental factors on the H/L ratio across bird communities in different habitats. While my study found no significant effect of precipitation on the H/L ratio in a forest bird community,

it is conceivable that bird communities in other habitat types may respond differently. For instance, Braem et al. (2023) demonstrated that agricultural landscapes in summer within temperate-zone regions often experience drier soils and higher temperature fluctuations compared to forests, potentially impacting ecological processes and the distribution of sensitive organisms. Consequently, it is plausible that bird communities in open landscapes could exhibit stronger physiological reactions (such as elevated H/L ratios) to changes in temperature and precipitation than those in forests, rendering them potentially more vulnerable to the effects of global warming and extreme weather events.

(2) Another compelling research question is whether birds in forests with human disturbance exhibit higher stress levels compared to those in undisturbed forests. Previous studies on single species have indicated that human activities, such as tourism, can act as stressors for forest birds (Rösner et al. 2014; Rösner et al. 2023) expanding this inquiry to bird communities can provide valuable insights into the broader impact of human activities, including walking dogs, hiking, biking, and horseback riding, on avian populations, thereby emphasizing the importance of maintaining undisturbed refuge areas within forests. A comparative approach, measuring the H/L ratio in both undisturbed and disturbed forest bird communities with similar community compositions and sampling periods, while also monitoring the frequency and types of disturbances in the latter, can elucidate the significance of undisturbed forest areas for bird community conservation efforts.

(3) Physiological stress measures can provide valuable insights into the impact of wind turbines on bird communities. While the installation of wind turbines is often discussed controversially, being both crucial for renewable energy goals and potentially harmful to birds due to collision or habitat degradation (Thaxter et al. 2017; Coppes et al. 2020; Rehling et al. 2023), studies like that of Rehling et al. (2023) have found a significant decrease in bird abundance in proximity to wind turbines in forests. To better understand this effect and determine the distance over which wind turbines affect birds, sampling birds at various distances from turbines and in turbine-free forests could be conducted. I hypothesise that birds closer to turbines may exhibit higher H/L ratios compared to those further away or in turbine-free areas. These results could inform decisions regarding the optimal proximity of wind farms to high-quality

habitats, aiming to mitigate the negative impact of turbines on forest bird communities.

(4) Evaluating the quality and relevance of stopover sites during bird migration through physiological stress measures, such as the H/L ratio, holds promise for understanding the factors influencing birds' stopover decisions. Previous studies have already shown that habitat composition determines stopover decisions in birds, and both water and forest availability were found to affect the birds' decisions (Bonter et al. 2009). While stopover sites are crucial for resting and refueling during long migratory journeys, their quality can vary significantly. Factors such as water availability, shrub availability, and the degree of human disturbance may all influence the suitability of stopover sites for birds. By monitoring the importance of these factors and their impact on bird stress levels, we can gain insights into what constitutes an optimal stopover site. For instance, if water availability is found to have a positive effect on birds' stress levels, this information could be used to identify and enhance existing stopover sites with adequate water resources or even create new suitable stopover sites along migratory routes. Ultimately, this research could contribute to the conservation and management of stopover habitats, ensuring that migrating birds have access to vital resources during their journeys. However, it is important to consider that bird migration is already a challenging season for birds, therefore the duration of the stopover and the muscle and fat reserves of the birds must be considered in such an analysis, and also the influence of intrinsic factors on the H/L ratio during migration needs to be evaluated first

7.3 Advancing Blood Cell Analysis: Expanding Applications of Deep Learning

To improve the identification of rare cell types such as basophils and monocytes, generating more training data could be crucial (Vogelbacher et al. 2024). A larger training dataset could significantly improve the precision of the neural network. Furthermore, expanding the approach to include bird species from diverse orders would increase its utility for ornithologists. As bird cell morphology varies across species and orders (Feldman et al. 2000), it is essential to train and validate the model for each new order, such as Procellariiformes, Sphenisciformes, Charadriiformes, or Psittaciformes, before application. This broader applicability could make avian blood smear evaluation more appealing for assessing

parameters such as the H/L ratio as a physiological stress response or leukocyte counts as indicators of immunity investment. Moreover, maintaining consistent counting quality improves the comparability of study results by reducing observer bias.

Incorporating haemosporidian blood parasites into the training data presents a valuable opportunity to further enhance the capabilities of the deep learning approach (Vogelbacher et al. 2024). While genetic lineage identification can be achieved through PCR, microscopy remains essential for verifying infections, identifying morphotypes, and determining parasitaemia levels. Research has shown that higher levels of parasitaemia can increase the costs of red blood cell production, thereby affecting the physiology and fitness of birds (Schoenle et al. 2017). However, manually evaluating morphotypes and parasitaemia levels using microscopy is both time-consuming and requires significant expertise. Fortunately, it is feasible to train the deep learning approach to identify morphotypes, count infected blood cells, and even differentiate the developmental stages of parasites. This advancement would streamline the evaluation of blood smears for blood parasites and enable the analysis of larger quantities of blood smears with minimal effort.

The automation of avian blood smear analysis further presents an opportunity for analysing large and historical collections of samples from universities and museums, where manual evaluation is impractical due to the sheer volume of samples. Evaluating older samples offers insights into changes in avian blood parasites and haematological parameters over time. Comparing historical data with recently collected samples enables the assessment of changes over longer time spans. Particularly when the sampling date and location of historical samples are known and habitat types have remained relatively stable, such comparisons can provide valuable insights into physiological adaptations over time.

Furthermore, the implementation of the new neural network (Vogelbacher et al. 2024) could facilitate the establishment of a database where scientists can share AI-generated counting results along with sample metadata, similar to the MalAvi database for avian malaria (Bensch et al., 2009). Such a database would increase the availability of comparative data, thereby enhancing the interpretation of results. Additionally, the accessibility of a large dataset on haematological parameters across multiple species, geographical regions, and habitats could serve as a valuable basis for meta-analysis. Such analyses could, for instance, identify variations in the H/L ratio attributed to different seasons, bird migration, habitat composition

(e.g., determined using remote sensing), or climatic conditions, particularly for species with broad distribution ranges.

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Appendices

Appendix Chapter II

Table S 2.4: List of the sampled avian families and species: Number of samples (n), prevalence of haemosporidian parasites (Prev.), number of individuals infected with *Haemoproteus* spp. (H), *Leucocytozoon* spp. (L), and *Plasmodium* ssp. (P), as well as the detected lineages as described in the MalAvi database (Bensch et al. 2009). Lineages with new host-parasite interactions are highlighted in bold and marked with ^{HP}, lineages that are novel to Germany are underlined and marked with ^{G}. In case of a host lineage interaction that was described in another country before but is new to Germany the lineage name is italic, which is marked with ^{HPG}.

Family/Species	**n**	**Prev.**	**H**	**L**	**P**	**Lineages detected**
Accipitridae	8	0%	-	-	-	
Buteo buteo	8	0%	-	-	-	-
Falconidae	1	0%	-	-	-	
Falco tinnunculus	1	0%	-	-	-	-
Columbidae	1	100%	-	1	-	
Columba palumbus	1	100%	-	1	-	L_AEMO02
Picidae	13	0%	-	-	-	
Dendrocopos major	11	0%	-	-	-	-
Dendrocopos medius	1	0%	-	-	-	-
Dryocopus martius	1	0%	-	-	-	-
Troglodytidae	17	5.9%	-	1	-	
Troglodytes troglodytes	17	5.1%	-	1	-	*L_PARUS20* ^{HPG}
Prunellidae	2	50%	1	-	-	
Prunella modularis	2	50%	1	-	-	H_DUNNO01 ^{G}
Muscicapidae	84	13.1%	4	4	3	
Erithacus rubecula	84	13.1%	4	4	3	*H_PARUS1* ^{HPG}, H_ROBIN1, L_BT2 ^{G}, L_PARUS20 ^{HP}, L_PARUS21 $^{HP, G}$, *L_SFC8* ^{HPG}, P_LINN1, *P_SGS1* ^{HPG}
Turdidae	53	94.3%	37	34	14	
Turdus philomelos	15	93.3%	9	11	3	H_CUKI1 $^{HP, G}$, H_TUPHI01, L_STUR1, L_TUPHI06 ^{G},

Family/Species	n	Prev.	H	L	P	Lineages detected
						L_TUPHI13 [G], L_TUPHI14 [G], *P_LINN1* [HPG], P_TURDUS1, H_undetermined, L_undetermined
Turdus viscivorus	3	100%	2	-	1	*H_CUKI1* [HPG, G], *P_SYAT05* [HPG]
Turdus merula	35	94.3%	26	23	10	H_TUCHR01 [G]. H_TURDUS2, L_NEVE01 [G], L_TUMER01 [G], P_LINN1, P_SYAT05, H_undetermined, L_undetermined
Sylviidae	56	50%	27	-	1	
Sylvia atricapilla	56	50%	27	-	1	*H_PARUS1* [HPG], H_SYAT01, H_SYAT02, H_SYAT03, H_SYAT14, *H_WW2* [HPG], P_AEMO01 [HP, G], H_undetermined
Phylloscopidae	23	4.3%	1	-	-	
Phylloscopus sibilatrix	10	10%	1	-	-	H_PHSIB2 [G]
Phylloscopus collybita	13	0%	-	-	-	-
Regulidae	1	0%	-	-	-	
Regulus ignicapilla	1	0%	-	-	-	-
Paridae	131	75.6%	59	93	-	
Parus major	65	83.1%	36	52	-	H_PARUS1, H_PHSIB1, L_PARUS4, L_PARUS7, L_PARUS16, L_PARUS18, L_PARUS19, L_PARUS20, L_PARUS22, L_undetermined
Periparus ater	3	66.7%	-	2	-	L_PARUS19, L_PARUS20 [HP]
Cyanistes caeruleus	27	96.3%	18	26	-	H_PARUS1, L_PARUS4, L_PARUS11, L_PARUS12,

Family/Species	n	Prev.	H	L	P	Lineages detected
						L_PARUS14, L_PARUS19, L_PARUS22, *L_PARUS33* [HPG], L_undetermined
Lophophanes cristatus	5	20%	-	1	-	L_PARUS4 [HP]
Poecile palustris	31	51.6%	5	12	-	*H_PARUS1* [HPG], *L_PARUS4* [HPG], *L_PARUS18* [HPG], *L_PARUS22* [HPG],
Sittidae	16	56.3%	7	2	-	
Sitta europaea	16	56.3%	7	2	-	H_PARUS1, L_PARUS20, *L_PARUS22* [HPG], L_undetermined
Certhiidae	25	0%	-	-	-	
Certhia familiaris	22	0%	-	-	-	-
Certhia brachydactyla	3	0%	-	-	-	-
Corvidae	12	75%	5	7	-	
Garrulus glandarius	7	100%	5	5	-	H_CIRCUM05, H_GAGLA02 [G], L_COCOR02 [G], L_GAGLA06 [G], H_undetermined
Corvus corone	5	40%	-	2	-	L_CCORAX03 [HP, G], L_CCORAX07 [HP, G]
Fringillidae	32	59.4%	14	4	-	
Fringilla coelebs	26	61.5%	15	2	-	H_CCF1, H_CCF2, H_CCF3 [G], H_CCF6 [G], H_CCF23 [G], L_BRAM3 [G]
Pyrrhula pyrrhula	3	33.3%	1	-	-	H_CCF23
Coccothraustes coccothraustes	3	66.7%	2	2	-	*H_HAWF6* [HPG, G], L_undetermined

Table S 2.5: Haemosporidian lineages that were either detected for the first time in the host species where it was detected in our study (HP) or that were detected for the first time in Germany (G) or the host-parasite interaction was new to Germany (HPG). The columns "Previously described host species" and "Previously described countries" refer to Europe as Geographical region (including Russia). The column "Host-Parasite country" shows where the host-parasite interaction was already described and includes all countries. The information about previously found interactions and the geographic distribution of lineages was taken from the MalAvi Database (Bensch et al. 2009).

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
Plasmodium				
AEMO01	*S. atricapilla* [HP, G]	*Aegypius monachus, Cossypha niveicapilla, Cyanomitra olivacea, Cyanomitra verticalis, Dicrurus adsimilis, Euplectes capensis, Ficedula albicollis, Ficedula hypoleuca, Gymnoris superciliaris, Hedydipna collaris, Merops apiaster, Nectarinia venusta, Ploceus ocularis, Ploceus princeps, Ploceus velatus, Pytilia melba, Quelea quelea, Sylvia borin, Sylvietta virens, Terpsiphone viridis*	Benin, Gabon, Hungary, Kenya, Malawi, Nigeria, Portugal, São Tomé and Príncipe, Spain, Sweden, United Kingdom	-
LINN1	*E. rubecula, T. merula, T. philomelos* [HPG]	*Athene noctua, Carduelis cannabina, Carduelis carduelis, Carduelis flammea, Corvus corone, Cyanistes caeruleus, Erithacus rubecula, Fratercula artica, Fulica cristata, Luscinia luscinia, Luscinia megarhynchos, Luscinia svecica, Passer montanus, Pyrrhula pyrrhula, Spheniscus demersus, Sylvia atricapilla, Turdus merula, Turdus philomelos*	Austria, Czech Republic, Germany, Hungary, Italy, Portugal, Slovakia, Spain, Sweden, Switzerland, United Kingdom	Austria, New Zealand, Portugal, Slovakia, Sweden
SGS1	*E. rubecula* [HPG]	*Acrocephalus agricola, Acrocephalus arundinaceus, Acrocephalus palustris, Acrocephalus schoenobaenus, Acrocephalus scirpaceus, Alauda*	Algeria, Armenia, Austria, Belgium, Benin, Bulgaria, Canada, China, Czech Republic,	Portugal, Russia, Slovakia, Spain, Sweden

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		arvensis, Amazilia chionogaster, Anas acuta, Athene noctua, Botaurus stellaris, Bubo scandiacus, Bubulcus ibis, Carduelis cannabina, Carduelis carduelis, Carduelis chloris, Carduelis spinus, Carpodacus erythrinus, Cercotrichas coryphoeus, Cercotrichas galactotes, Cercotrichas podobe, Certhia brachydactyla, Certhia familiaris, Cettia cetti, Ciconia ciconia, Coccothraustes coccothraustes, Colibri coruscans, Columba livia, Conirostrum cinereum, Corvus corax, Corvus corone, Corvus macrorhynchos, Corvus monedula, Cracticus tibicen, Cyanistes caeruleus, Cyanistes teneriffae, Cyanopica cooki, Delichon urbicum, Emberiza calandra, Emberiza cia, Emberiza cirlus, Emberiza citrinella, Emberiza elegans, Emberiza godlewskii, Emberiza hortulana, Emberiza tahapisi, Erithacus rubecula, Estrilda astrild, Euplectes orix, Ficedula albicollis, Ficedula hypoleuca, Fratercula arctica, Fringilla coelebs, Gallinago gallinago, Gallus gallus, Garrulus glandarius, Grus japonensis, Grus leucogeranus, Grus nigricollis, Grus vipio, Himantopus himantopus, Hippolais polyglotta, Hypsipetes amaurotis, Lamprotornis	Egypt, Falkland Islands, France, Germany, Greece, Hungary, India, Israel, Italy, Japan, Kenya, Korea South, Lithuania, Mongolia, Morocco, Netherlands, New Zealand, Nigeria, Norway, Peru, Poland, Portugal, Romania, Russia, Serbia, Slovakia, South Africa, Spain, Sweden, Switzerland, Tunisia, Turkey, Ukraine, United Kingdom, United States	

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		hildebrandti, Lamprotornis superbus, Lanius collaris, Larosterna inca, Larus argentatus, Larus cachinnans, Larus mongolicus, Lophophanes cristatus, Lophophorus impejanus, Loxia curvirostra, Luscinia luscinia, Luscinia svecica, Marmaronetta angustirostris, Microscelis amaurotis, Monticola saxatilis, Motacilla flava, Muscicapa striata, Oenanthe oenanthe, Pachyptila belcheri, Parus major, Parus palustris, Parus venustulus, Passer domesticus, Passer domesticus x hispaniolensis, Passer griseus, Passer hispaniolensis, Passer luteus, Passer melanurus, Passer montanus, Passer rufocinctus, Perdix perdix, Periparus ater, Phleocryptes melanops, Phoenicurus moussieri, Phoenicurus ochruros, Phoenicurus phoenicurus, Phylloscopus trochilus, Pica pica, Ploceus capensis, Ploceus melanocephalus, Ploceus velatus, Poecile montanus, Poecile varius, Prunella modularis, Pycnonotus capensis, Pyrrhula pyrrhula, Pytilia melba, Riparia riparia, Saxicola maura, Saxicola rubetra, Sayornis nigricans, Serinus canaria, Serinus serinus, Serpophaga cinerea, Sitta europaea, Somateria spp, Sphenisciformes spp, Spheniscus		

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		demersus, Spheniscus humboldti, Sturnus cineraceus, Sturnus tristis, Sturnus unicolour, Sylvia atricapilla, Sylvia borin, Sylvia communis, Sylvia curruca, Sylvia deserticola, Sylvia melanocephala, Sylvia nisoria, Sylvia undata, Tachycineta bicolour, Tragopan temminckii, Troglodytes aedon, Troglodytes troglodytes, Turdus merula, Turdus viscivorus, Zonotrichia capensis		
SYAT05	*T. merula, T. viscivorus* [HPG]	*Alauda arvensis, Anthornis melanura, Cettia cetti, Cinclus cinclus, Cyanistes caeruleus, Emberiza citrinella, Erithacus rubecula, Ficedula parva, Fringilla coelebs, Hemiphaga novaeseelandiae, Lamprotornis hildebrandti, Lamprotornis superbus Meleagris gallopavo, Melopsittacus undulates, Parus major, Passer domesticus, Petroica australis, Petroica macrocephala, Pipile jacutinga, Prunella modularis, Saxicola maura, Sturnus unicolour, Sylvia atricapilla, Sylvia borin, Sylvia melanocephala, Turdus merula, Turdus migratorius, Turdus pelios, Turdus philomelos, Turdus viscivorus, Zosterops lateralis, Zoothera dauma*	Armenia, Austria, Brazil, Bulgaria, Gabon, Germany, Hungary, Italy, Japan, Kenya, Morocco, Netherlands, New Zealand, Portugal, Russia, Serbia, Slovakia, Spain, Sweden, Switzerland, United States	Armenia, Austria, Bulgaria, Germany, Hungary, Iran, Italy, Morocco, New Zealand, Portugal, Russia, Slovakia, Spain, Sweden, Switzerland
TURDUS1	*T. philomelos*	*Accipiter gentilis, Accipiter gularis, Accipiter nisus, Acrocephalus paludicola, Acrocephalus schoenobaenus, Aegolius funereus,*	Austria, Bulgaria, China, Czech Republic, Finland, France, Germany,	Austria, Bulgaria, Germany,

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		Anthus hodgsoni, Anthus trivialis, Buteo buteo, Carpodacus erythrinus, Coccothraustes coccothraustes, Corvus monedula, Cyanistes caeruleus, Delichon urbicum, Emberiza citrinella, Emberiza schoeniclus, Erithacus rubecula, Ficedula albicollis, Ficedula hypoleuca, Ficedula parva, Fringilla coelebs, Hirundo rustica, Luscinia cyanura, Luscinia luscinia, Luscinia svecica, Motacilla flava, Muscicapa dauurica, Parus major, Parus palustris, Passer domesticus, Periparus ater, Philomachus pugnax, Phoenicurus phoenicurus, Phylloscopus collybita, Phylloscopus trochilus, Poecile montanus, Prunella modularis, Pyrrhula pyrrhula, Regulus regulus, Sitta europaea, Sylvia atricapilla, Sylvia borin, Sylvia communis, Troglodytes troglodytes, Turdus merula, Turdus philomelos	Japan, Lithuania, Netherlands, Nigeria, Norway, Poland, Russia, Slovakia, Spain, Sweden, Switzerland, Turkey, United Kingdom	Slovakia, Sweden
Haemoproteus				
CCF1	*F. coelebs*	*Emberiza cirlus, Fringilla coelebs*	Bulgaria, Germany, Lithuania, Morocco, Portugal, Sweden	Bulgaria, Germany, Lithuania, Morocco, Portugal, Sweden
CCF2	*F. coelebs*	*Cyanistes caeruleus, Emberiza cirlus, Erithacus rubecula, Ficedula semitorquata, Fringilla coelebs, Fringilla montifringilla, Riparia*	Armenia, Azerbaijan, Bulgaria, Germany,	Armenia, Azerbaijan, Germany, Lithuania,

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		riparia, Serinus serinus, Sylvia atricapilla	Lithuania, Morocco, Portugal, Russia, Serbia, Slovakia, Spain, Sweden	Morocco, Portugal, Russia, Serbia, Slovakia, Sweden
CCF3	*F. coelebs* [G]	*Carduelis flammea, Carpodacus erythrinus, Chloris chloris, Fringilla coelebs, Pyrrhula pyrrhula*	Czech Republic, Russia, Serbia, Slovakia, Sweden	Russia, Slovakia, Sweden
CCF6	*F. coelebs* [G]	*Cyanistes caeruleus, Fringilla coelebs, Parus palustris*	Bulgaria, Portugal, Russia, Serbia, Slovakia, Sweden	Bulgaria, Portugal, Russia, Serbia, Slovakia, Sweden
CCF23	*F. coelebs* [G], *P. pyrrhula* [HP, G]	*Fringilla coelebs*	Portugal, Slovakia, Sweden	Portugal, Slovakia, Sweden
CIRCUM05	*G. glandarius* [HPG]	*Corvus corax, Corvus corone, Garrulus glandarius, Pica pica*	Bulgaria, Germany, Italy, Portugal, Slovakia	Portugal, Slovakia
CUKI1	*T. philomelos* [HP, G], *T. viscivorus* [G]	*Turdus viscivorus*	Lithuania	Lithuania
DUNNO01	*P. modularis* [G]	*Emberiza citrinella, Prunella modularis*	United Kingdom, Slovakia, Sweden	United Kingdom, Slovakia, Sweden
GAGLA02	*G. glandarius* [G]	*Garrulus glandarius*	Bulgaria, Portugal, Serbia, Slovakia	Bulgaria, Portugal, Serbia, Slovakia
HAWF6	*C. coccothraustes* [G]	*Coccothraustes coccothraustes, Turdus merula*	Portugal, Slovakia	Slovakia
PARUS1	*C. caeruleus, E. rubecula* [HPG], *P. major, P. palustris*	*Acrocephalus schoenobaenus, Cyanistes caeruleus, Cyanistes teneriffae, Emeriza schoeniclus, Erithacus rubecula, Ficedula*	Armenia, Austria, Belgium, Finland, France, Germany, Lithuania, Poland,	Armenia, Austria, Azerbaijan, Belgium,

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
	[HPG], S. europaea, S. atricapilla [HPG]	*hypoleuca, Hippolais icterina, Panurus biarmicus, Parus major, Periparus ater, Phylloscopus collybita, Phylloscopus trochilus, Poecile montanus, Poecile palustris, Pyrrhula pyrrhula, Saxicola rubetra, Sitta europaea, Sylvia atricapilla, Sylvia borin, Sylvia communis, Sylvia curruca*	Portugal, Russia, Slovakia, Spain, Sweden, Switzerland	Finland, France, Germany, Iran, Lithuania, Morocco, Poland, Portugal, Russia, Slovakia, Spain, Sweden, Switzerland, United Kingdom
PHSIB1	*P. major*	*Acrocephalus arundinaceus, Acrocephalus schoenobaenus, Aegithalos caudatus, Cettia diphone, Corvus monedula, Cyanistes caeruleus, Emberiza schoeniclus, Erithacus rubecula, Ficedula albicollis, Ficedula hypoleuca, Fringilla coelebs, Luscinia calliope, Luscinia luscinia, Muscicapa striata, Panurus biarmicus, Parus major, Parus palustris, Phoenicurus phoenicurus, Phylloscopus collybita, Phylloscopus humei, Phylloscopus proregulus, Phylloscopus sibilatrix, Phylloscopus trochilus, Poecile montanus, Saxicola rubetra, Sitta europaea*	Finland, France, Germany, Japan, Kyrgyzstan, Lithuania, Netherlands, Nigeria, Poland, Russia, Slovakia, Spain, Sweden, Switzerland, United Kingdom	France, Germany, Russia, Sweden, Switzerland

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
PHSIB2	*P. sibilatrix* [G]	*Fringilla coelebs, Phylloscopus sibilatrix*	Lithuania, Slovakia, Sweden	Lithuania, Slovakia, Sweden
ROBIN1	*E. rubecula*	*Acrocephalus schoenobaenus, Alcedo atthis, Certhia familiaris, Cyanistes caeruleus, Erithacus rubecula, Luscinia luscinia, Luscinia megarhynchos, Parus palustris, Prunella modularis, Saxicola rubetra, Sylvia atricapilla, Turdus iliacus*	Bulgaria, Germany, Lithuania, Morocco, Nigeria, Portugal, Russia, Serbia, Slovakia, Spain, Sweden, Turkey	Bulgaria, Germany, Lithuania, Morocco, Portugal, Russia, Serbia, Slovakia, Spain, Sweden
SYAT01	*S. atricapilla*	*Cyanistes caeruleus, Pseudoalcippe abyssinica, Sylvia atricapilla, Sylvia borin, Sylvia communis*	Armenia, Belgium, France, Germany, Lithuania, Morocco, Portugal, Russia, Serbia, Slovakia, Spain, Sweden, Tanzania	Armenia, Belgium, France, Germany, Lithuania, Morocco, Portugal, Russia, Serbia, Slovakia, Spain, Sweden
SYAT02	*S. atricapilla*	*Cyanistes caeruleus, Luscinia megarhynchos, Melaenornis edolioides, Sylvia atricapilla*	Belgium, Benin, France, Germany, Morocco, Portugal, Russia, Serbia, Slovakia, Spain, Sweden	Belgium, France, Germany, Morocco, Portugal, Russia, Serbia, Slovakia, Spain, Sweden
SYAT03	*S. atricapilla*	*Cyanistes caeruleus, Erithacus rubecula, Ficedula albicollis, Parus major, Sylvia atricapilla, Sylvia borin, Troglodytes troglodytes*	Belgium, Germany, Portugal, Russia, Slovakia, Spain, Sweden	Belgium, Germany, Portugal, Russia, Slovakia, Spain, Sweden

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
SYAT14	*S. atricapilla*	*Sylvia atricapilla*	Germany, Portugal, Slovakia, Spain, Sweden	Germany, Portugal, Slovakia, Spain, Sweden
TUCHR01	*T. merula* [G]	*Turdus chrysolaus, Turdus iliacus, Turdus merula, Turdus philomelos, Turdus pilaris*	Austria, Russia, Sweden	Austria
TUPHI01	*T. philomelos* [HPG]	*Carduelis spinus, Cyanoramphus auriceps, Cyanoramphus novaezelandiae, Erithacus rubecula, Melopsittacus undulatus, Neophema pulchella, Neopsephotus bourkii, Phylloscopus collybita, Platycercus elegans, Platycerus icterotis, Polytelis alexandrae, Polytelis swansonii, Psitteuteles goldiei, Turdus merula, Turdus philomelos*	Austria, Bulgaria, Denmark, Germany, Lithuania, Portugal, Russia, Serbia, Slovakia, Sweden, Switzerland, United Kingdom	Austria, Bulgaria, Lithuania, Portugal, Russia, Serbia, Slovakia, Sweden
TURDUS2	*T. merula*	*Acrocephalus scirpaceus, Bolborhynchus lineola, Cardellina pusilla, Charmosyna papou, Cyanistes caeruleus, Cyanoramphus sp., Emberiza schoeniclus, Erithacus rubecula, Garrulus glandarius, Hippolais icterina, Ixoreus naevius, Junco hyemalis, Lanius collurio, Loxia leucoptera, Muscicapa striata, Myiopsitta monachus, Neophema pulchella, Panurus biarmicus, Parus major, Platycercus elegans, Polytelis alexandrae, Psitteuteles goldiei, Setophaga striata, Sylvia atricapilla, Turdus fulviventris, Turdus iliacus, Turdus merula, Turdus migratorius, Turdus*	Armenia, Austria, Bulgaria, China, Germany, Iran, Italy, Lithuania, Morocco, Peru, Portugal, Russia, Serbia, Slovakia, Sweden, United Kingdom, United States	Armenia, Austria, Germany, Iran, Italy, Lithuania, Morocco, Portugal, Russia, Serbia, Slovakia, Sweden

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		philomelos, Turdus pilaris, Turdus rubrocanus, Turdus serranus, Turdus torquatus		
WW2	*Sylvia atricapilla* [HPG]	*Acrocephalus arundinaceus, Acrocephalus palustris, Acrocephalus schoenobaenus, Acrocephalus scirpaceus, Certhia familiaris, Corvus monedula, Crex crex, Cyanistes caeruleus, Erithacus rubecula, Ficedula albicollis, Ficedula hypoleuca, Hirundo rustica, Jynx torquilla, Locustella naevia, Luscinia luscinia, Luscinia svecica, Panurus biarmicus, Parus major, Periparus ater, Phoenicurus phoenicurus, Phylloscopos collybita, Phylloscopos sibilatrix, Phylloscopos trochilus, Poecile palustris, Remiz pendulinus, Saxicola rubetra, Sylvia atricapilla, Sylvia borin, Sylvia communis, Sylvia curruca, Troglodytes troglodytes*	Bulgaria, Czech Republic, France, Germany, Italy, Lithuania, Norway, Poland, Portugal, Russia, Slovakia, Spain, Sweden, Switzerland, Ukraine	Bulgaria, Russia, Slovakia, Sweden
Leucocytozoon				
AEMO02	*C. palumbus*	*Aegypius monachus, Columba livia, Columba palumbus, Streptopelia turtur*	Germany, Greece, Italy, Portugal, Spain, Sweden	Germany, Portugal, Sweden
BRAM3	*F. coelebs* [G]	*Fringilla coelebs, Fringilla montifringilla*	Slovakia, Sweden	Morocco, Slovakia, Sweden
BT2	*E. rubecula* [HPG]	*Accipiter gentilis, Aegithalos caudatus, Aegolius funereus, Anthus trivialis, Carpodacus erythrinus, Erithacus rubecula, Hippolais icterina, Lanius collurio,*	Czech Republic, Germany, Italy, Norway, Russia, Slovakia, Spain, Sweden,	Slovakia, Sweden

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
		Locustella naevia, Luscinia svecica, Muscicapa striata, Parus major, Passer domesticus, Phoenicurus phoenicurus, Phylloscopus collybita, Phylloscopus sibilatrix, Phylloscopus trochilus, Prunella modularis, Regulus regulus, Saxicola rubetra, Sylvia atricapilla, Sylvia borin, Sylvia curruca	Switzerland, Ukraine	
CCORAX03	*C. corone* [HP, G]	*Corvus corax*	Bulgaria	-
CCORAX07	*C. corone* [HP, G]	*Corvus corax*	Bulgaria	-
COCOR02	*G. glandarius* [G]	*Corvus corax, Garrulus glandarius*	Bulgaria, Slovakia	Slovakia
GAGLA06	*G. glandarius* [G]	*Corvus corax, Garrulus glandarius*	Bulgaria, Portugal, Slovakia	Portugal, Slovakia
NEVE01	*T. merula* [G]	*Turdus merula*	Austria, Slovakia	Austria, Slovakia
PARUS4	*C. caeruleus, L. cristatus* [HP], *P. major, P. palustris* [HPG]	*Cyanistes caeruleus, Erithacus rubecula, Parus major, Periparus ater, Phylloscopus sibilatrix, Poecile palustris, Sylvia atricapilla, Sylvia communis*	Belgium, France, Germany, Portugal, Russia, Slovakia, Spain, Sweden, Switzerland, United Kingdom	Armenia, Azerbaijan, Belgium, France, Germany, Morocco, Portugal, Russia, Slovakia, Spain, Sweden, Switzerland, United Kingdom
PARUS7	*P. major*	*Cyanistes caeruleus, Luscinia svecica, Parus major, Periparus ater, Sitta europaea*	Germany, Japan, Portugal, Serbia, Slovakia, Spain, Sweden, United Kingdom	Germany, Portugal, Slovakia, Sweden

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
PARUS11	*C. caeruleus*	*Cyanistes caeruleus, Cyanistes teneriffae, Parus major*	France, Germany, Morocco, Portugal, Sweden	France, Germany, Portugal, Sweden
PARUS12	*C. caeruleus*	*Cyanistes caeruleus*	France, Germany, Portugal, Sweden	France, Germany, Portugal, Sweden
PARUS14	*C. caeruleus*	*Cyanistes caeruleus, Parus major, Sylvia atricapilla*	France, Germany, Portugal, Slovakia, Sweden, United Kingdom	France, Germany, Portugal, Slovakia, Sweden, United Kingdom
PARUS16	*P. major*	*Cyanistes caeruleus, Parus major, Sylvia atricapilla*	Armenia, Azerbaijan, France, Germany, Morocco, Portugal, Russia, Slovakia, Sweden, Switzerland, United Kingdom	Armenia, Azerbaijan, France, Germany, Morocco, Portugal, Russia, Slovakia, Sweden, Switzerland, United Kingdom
PARUS18	*P. major, P. palustris* [HPG]	*Cyanistes caeruleus, Parus major, Poecile palustris*	France, Germany, Portugal, Slovakia, Sweden, Switzerland, United Kingdom	France, Germany, Portugal, Sweden, Switzerland, United Kingdom

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
PARUS19	*C. caeruleus, P. ater, P. major*	*Cyanistes caeruleus, Cyanistes teneriffae, Delichon urbicum, Parus major, Periparus ater*	Armenia, Azerbaijan, Belgium, France, Germany, Morocco, Portugal, Slovakia, Spain, Sweden, Switzerland, United Kingdom	Armenia, Azerbeijan, Belgium, France, Germany, Morocco, Portugal, Slovakia, Spain, Sweden, Switzerland, United Kingdom
PARUS20	*E. rubecula* [HP], *P. ater* [HP], *P. major, S. europaea, T. troglodytes* [HPG]	*Cyanistes caeruleus, Luscinia svecica, Parus major, Poecile montanus, Poecile palustris, Sitta europaea, Troglodytes troglodytes*	France, Germany, Portugal, Serbia, Slovakia, Spain, Sweden, Switzerland, United Kingdom	France, Germany, Portugal, Slovakia, Sweden, Switzerland, United Kingdom
PARUS21	*E. rubecula* [HP, G]	*Cinclus cinclus, Cyanistes caeruleus, Cyanistes teneriffae, Emberiza schoeniclus, Luscinia svecica, Parus major, Passer domesticus, Sitta europaea, Sylvia melanocephala, Troglodytes troglodytes*	France, Morocco, Portugal, Spain, Sweden, United Kingdom	-
PARUS22	*C. caeruleus, P. major, P. palustris* [HPG], *S. europaea* [HPG]	*Certhia brachydactyla, Certhia familiaris, Cyanistes caeruleus, Lophophanes cristatus, Parus major, Poecile montanus, Poecile palustris*	France, Germany, Portugal, Russia, Serbia, Slovakia, Sweden, Switzerland, United Kingdom	Armenia, Slovakia
PARUS33	*C. caeruleus* [HPG]	*Cyanistes caeruleus, Parus major*	France, Germany, Switzerland	France

Parasite lineage	Host species	Previously described host species	Previously described countries	Host – parasite country
SFC8	*E. rubecula* [HPG]	*Acrocephalus schoenobaenus, Dendrocopos major, Eremophila alpestris, Erithacus rubecula, Ficedula parva, Luscinia megarhynchos, Muscicapa striata, Phoenicurus phoenicurus, Phylloscopus brehmi, Phylloscopus collybita, Phylloscopus trochilus, Sitta europaea, Sitta krueperi, Sylvia atricapilla, Sylvia borin, Sylvia cantillans, Sylvia conspicillata, Sylvia melanocephala, Troglodytes troglodytes*	Armenia, China, France, Germany, Morocco, Nigeria, Portugal, Russia, Slovakia, Spain, Sweden	Armenia, Portugal, Slovakia, Sweden
STUR1	*T. philomelos*	*Erithacus rubecula, Turdus philomelos*	Germany, Lithuania, Portugal, Slovakia	Germany, Lithuania, Portugal, Slovakia
TUMER01	*T. merula* [G]	*Passer domesticus, Turdus merula*	Austria, Portugal, Spain, Sweden	Austria, Morocco, Portugal, Spain, Sweden
TUPHI06	*T. philomelos* [G]	*Turdus merula, Turdus philomelos*	Austria, Portugal, Slovakia, Sweden	Austria, Portugal, Slovakia, Sweden
TUPHI13	*T. philomelos* [G]	*Turdus philomelos*	Austria, Russia, Slovakia	Austria, Russia, Slovakia
TUPHI14	*T. philomelos* [G]	*Turdus philomelos*	Slovakia	Slovakia

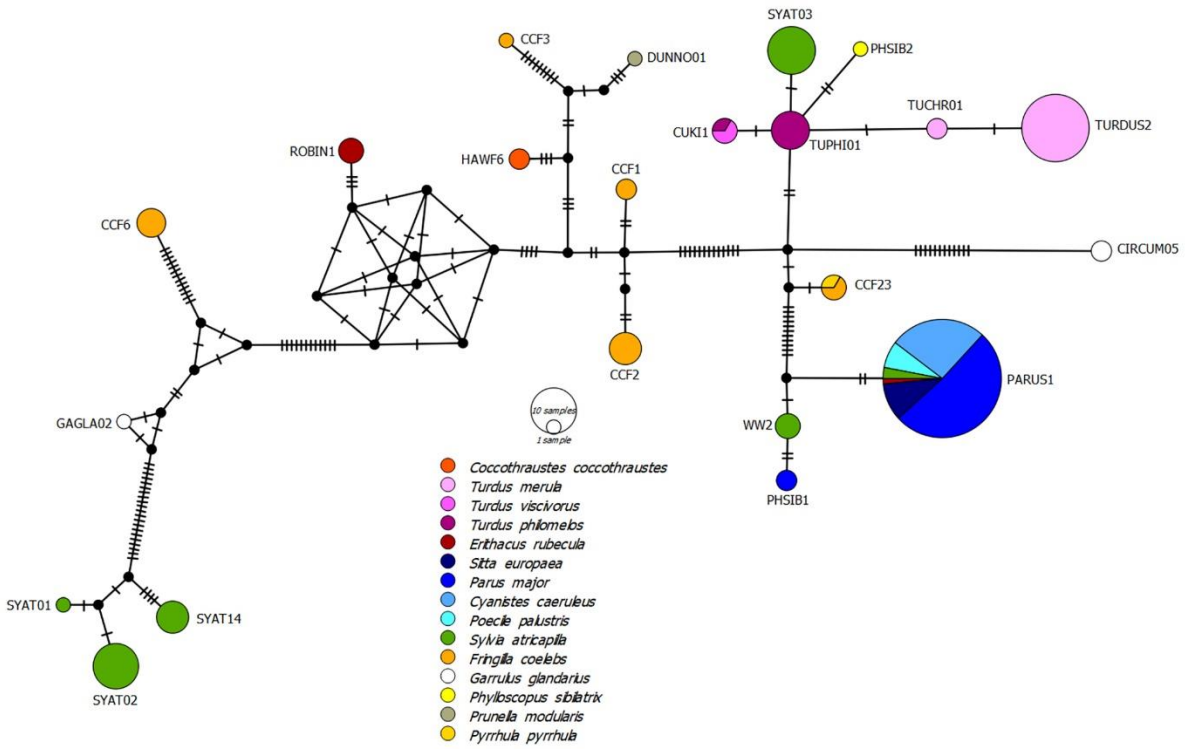

Figure S 2.4: A median-joining network of the mitochondrial cytochrome b lineages of Haemoproteus spp. (480 bp fragment, Hellgren et al. 2004). Each circle represents one lineage. The size of the circle indicates the frequencies of the lineages and the colours show in which host species the lineage was found. Hatch marks represent number of mutations between lineages.

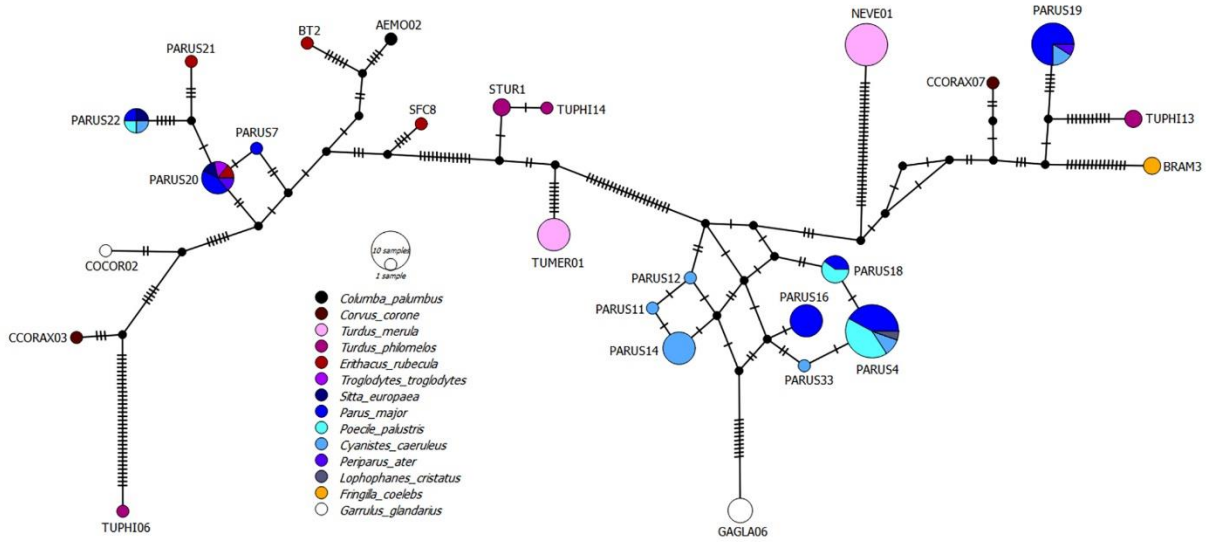

Figure S 2.5: A median-joining network of the mitochondrial cytochrome b lineages of Leucocytozoon spp. (478 bp fragment, Hellgren et al. 2004). Each circle represents one lineage. The size of the circle

indicates the frequencies of the lineages and the colours show in which host species the lineage was found. Hatch marks represent the number of mutations.

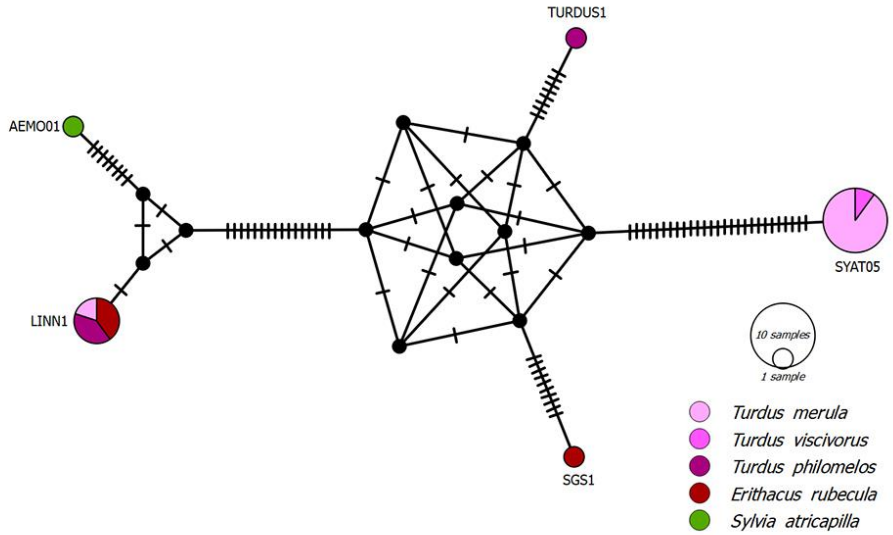

Figure S 2.6: A median-joining network of the mitochondrial cytochrome b lineages of Plasmodium spp. (480 bp fragment, Hellgren et al. 2004). Each circle represents one lineage. The size of the circle indicates the frequencies of the lineages and the colours show in which host species the lineage was found. Hatch marks represent the number of mutations.

Appendix Chapter IV

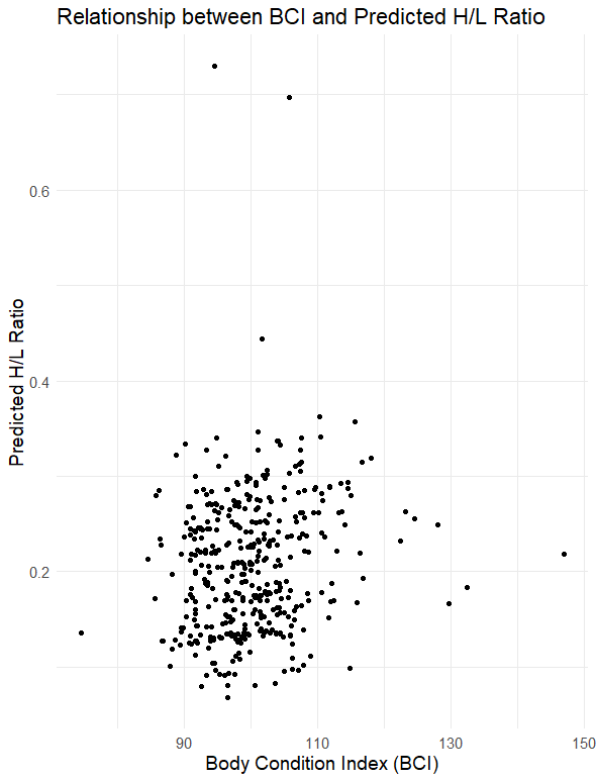

Figure S 4.4: The relationship between Body Condition Index (BCI) and Heterophil/Lymphocyte (H/L) Ratio, based on data from the first model in Chapter IV.

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Curriculum Vitae

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Declaration / Erklärung

Ich versichere, dass ich meine Dissertation mit dem Titel „Exploring Endoparasites and Physiological Stress: Insights from a European Forest Bird Community" selbstständig und ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keinr anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 15.04.2024

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Finja Strehmann