The Nature of Cancer: Unifying Evolutionary Theory in Cancer Biology

by

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ABSTRACT

Evolutionary theory provides a rich framework for understanding cancer dynamics across scales of biological organization. The field of cancer evolution has largely been divided into two domains, comparative oncology - the study of cancer across the tree of life, and tumor evolution. This work provides a theoretical framework to unify these subfields with the intent that an understanding of the evolutionary dynamics driving cancer risk at one scale can inform the understanding of the dynamics on another scale. The evolution of multicellular life and the unique vulnerabilities in the cellular mechanisms that underpin it explain the ubiquity of cancer prevalence across the tree of life. The breakdown in cellular cooperation and communication that were required for multicellular life define the hallmarks of cancer. As divergent life histories drove speciation events, it similarly drove divergences in fundamental cancer risk across species. An understanding of the impact that species' life history theory has on the underlying network of multicellular cooperation and somatic evolution allows for robust predictions on cross-species cancer risk. A large-scale veterinary cancer database is utilized to validate many of the predictions on cancer risk made from life history evolution. Changing scales to the cellular level, it lays predictions on the fate of somatic mutations and the fitness benefits they confer to neoplastic cells compared to their healthy counterparts. The cancer hallmarks, far more than just a way to unify the many seemingly unique pathologies defined as cancer, is a powerful toolset to understand how specific mutations may change the fitness of somatic cells throughout carcinogenesis and tumor progression. Alongside highlighting the significant advances in evolutionary approaches to cancer across scales, this work provides a lucid confirmation that an understanding of both scales provides the most complete portrait of evolutionary cancer dynamics.

ACKNOWLEDGMENTS

First, I hope the reader knows how consciously aware I am of the absurdity and privilege that I have enjoyed being able to dedicate so much of my life to studying the single topic that captivates me the most. Should the reader themselves feel compelled to follow a similar path, the following people were indispensable to me. I am deeply thankful that such an effort was put forth by my father and grandfather to instill in me a terrific wonder for the natural world. For any would- be biologist this is as good a starting point as one could hope for. Some significant credit for any decent writing found here should be given to my mother, who insisted from a young age to incorporate reading as a daily practice. Another motivation to include this acknowledgement section was fueled by fear that otherwise Carlo Maley may not explicitly know how much I admire him. The opportunities and guidance he provided me could never be repaid but they will at least be paid forward. The intellectual breadth and depth of my remaining committee members; Athena Aktipis, Aurora Nedelcu, Kenneth Beutow, and Carolyn Compton is a gift I feel constantly unworthy of. To be mentored by any one of them individually would be an incredible advance for someone's career, but to have them all assembled for my committee was invaluable. To have a friend of superior intellect is a constant source of annoyance and motivation, so I am thankful for my many late-night discussions with Hunter McCollum. Lastly, Danielle Martin, an enduring light through the darker corners of this journey and a constant guardrail against the worst of my own tendencies. For all those listed above and others, I am grateful.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

Figure Page

PREFACE

My hope is that the addition of the introductory chapter, *The Nature of Cancer Evolution,* to my formal dissertation will serve as a scaffold for both understanding the scientific manuscripts included and what my intentions were for choosing the work I pursued. The language and framework used in this introduction is also employed in the chapter introductions I attached ahead of each of the manuscripts included as part of this thesis. This work rests on the shoulders of giants, many of whom are members of my thesis committee. But each of these chapters also rests on a complicated prelude, which I take the time in the chapters' introductions to address and clarify. For all the ways cancer evolution is distinct among scientific fields, it is alike with the rest in that many of its advances are predicated on arguments and infighting. I intend to use these introductions to show my appreciation for these arguments and where I lie within them. Therefore, the chapter introductions are largely without citations, the criticisms I address and those I propose should be generally applicable without having to point the finger at any specific publication. These introductory spaces are also used to address important intersections that this work makes with the history of science and society. Lastly, as much as this work was an evolutionary theorist's investigation into cancer biology, it was equally a cancer biologist's investigation into evolutionary theory. Although cancer is often a tale of tragedy and loss, it is also the story of us. A story that unites humankind with so much of the life on this planet and a constant reminder of our lowly origins.

CHAPTER 1

THE NATURE OF CANCER EVOLUTION

The Natural History of Cancer

The natural history of cancer sets the prologue to the permanence of the disease in multicellular life and offers insights into how we may ultimately gain a clinical governance over it. Recent paleontological discoveries extend our knowledge of the pervasiveness of clinically distinguishable cancers (CDCs) in non-humans to the Jurassic era (Rothschild et al., 1999). Additionally, archaeological surveys demonstrate, at least to some extent, that cancer in humans reaches out to prehistory (David & Zimmerman, 2010; Marques et al.,2022; Prates et al., 2011). The radical changes in Western lifestyle mixed with novel carcinogenic factors humans are exposed to in the past 100 years have led many to believe cancer is a principal side effect of a mismatched environment. While these shifts in environmental factors certainly have modulated humans' cancer risk (Greaves & Aktipis, 2016; Hochberg & Noble, 2017) some clarification is required here. Before we can fully digest the evolutionary history of cancer across species we must land on some agreeable points on its history in humans. These assumptions can allow us to avert much of the arguments over the complex genotype-environment interactions that determine both individual level and species level cancer risk. Cancer can be measured across species in two factors. The binary – species does or does not get cancer – and then the more epidemiological measurements of prevalence of the disease in the

population. To appreciate the evolutionary history of cancer and its impact on disease risk across species we have to first agree on the binary measurement, humans have some baseline lifetime risk of cancer. If we were to freeze a moment in evolutionary history to the transition from our most recent bipedal ancestor to anatomically modern humans that population would have *some*, albeit undetermined, risk of developing cancer. This is true even if factors like predation, infant mortality, and disease precluded that risk from ever being actualized.

We need not ever find fossil evidence of tumors in early hominids to know this to be true. The theoretical framework from multicellular cooperation and somatic evolution points to its legitimacy (A. Aktipis, 2016; Aktipis C. Athena et al., 2015; Michod & Nedelcu, 2003; Trigos et al., 2018). A counter to my own argument here is that there is a plethora of the non-cancer pathologies that stem from somatic dysfunction, originating ultimately from breakdowns in multicellular cooperation that are like those found in cancer. That being true, the fitness of healthy cells is so drastically lower than that of somatic cells harboring oncogenic mutations that cancer should be seen as inevitable at the population level(Di Gregorio et al., 2016). Of course, once we have landed this conceptual baseline of cancer risk in humans it takes little leap in logic to apply it to non-human species. In the introduction to *Cancer Across Vertebrates* I address many of the criticisms brought against the use of zoo data in accurately determining cross-species cancer risk.

Making Sense of Cancer with Evolution

Evolution offers a terrific complex of theoretical and quantitative tools to explore nearly all biological questions. While standard organismal biology has long embraced these approaches, it is only in recent decades that the massive amounts of public funding and focus on human disease have begun to agree with the late Dobzhansky's adage. Evolutionary theory not only describes processes such as carcinogenesis and tumor subclone evolution, it also provides an explanatory framework for the intrinsic cancer risk across species.

A plethora of scientific publications and proposals in the past decade have leaned into a phrase that manifests in a common way; *evolutionary theory can be used to understand tumor dynamics and therapeutic resistance.* But this implies special usages of evolutionary theory, or that tumor evolution serves as some microcosm of evolution. At worst, it implies that evolutionary theory serves as some interpretive framework for tumor dynamics. These processes are not some abstract manifestation of evolutionary mechanisms but rather *they are evolution.* The mechanisms of evolution exist and churn without any imperative scale. The same evolutionary processes that brought upon the diversification in the finch species of the Galapagos, or drives the ever-problematic interactions between viral agents and humans, are the same processes that dictate cancer initiation and the selective forces that shape the emergence of resistance.

Similar language has been invoked when discussing my work implementing phylogenetic comparative methods (PCMs) to study cancer risk as a species' trait. In the sense of applying the central methods from each discipline,

tumor evolution is not a particularly unique evolutionary system, nor is cancer risk a particularly unique species' trait. This clarification may seem like semantics but it opens the door to simple truths that widen the approaches we may take to understand cancer biology. Two of these truths should be most apparent. First, there should be limited instances of theoretical or quantifiable approaches from organismal evolutionary biology that cannot be translated to study cancer biology. Measurements of complex ecological interactions, modes of diversification, identification of selective pressures and their respective strengths, and the rich depth of comparative phylogenetics should be relevant in tumor evolution. Secondly, as with all truths, the inverse should also be true. If all of evolutionary theory can be utilized to study cancer biology, then cancer biology should be a relevant model to understand evolutionary biology. Further, this should remain true across the relevant scales of cancer biology, specifically when we cross scales from tumor evolution to the evolution of intrinsic cancer risk across species. In applying evolutionary methods to these questions we should be just as prepared to make discoveries about evolutionary biology as we are to make discoveries about cancer biology.

The meeting proceeds from all the major cancer research societies starting in the 1990s emerged with a unifying theme; *cancer is a complex disease.* This sentiment, and the unhelpful condescension it implies, highlights the desperate need for an evolutionary-ecology framework for cancer biology. It is difficult to get within earshot of ecologist turned cancer biologist Dr. Joel Brown without hearing that, of course, "cancer is no more complex than any other

ecological network, perhaps even far less complex than some found in your own backyard". As the publications describing the ecology of tumors amass, his statement continues to take on a deeper relevance.

Despite the preeminence of evolutionary theory in cancer biology it wasn't described as an evolutionary system until Peter Nowell's somewhat understated 1976 *Science* article. As thinking about cancer as an evolutionary process continued to build in the early 2000s, one of the most famous frameworks for understanding cancer was simultaneously being constructed. I have often wondered who has the better perspective, the cancer biologist that adopts the evolutionary approach to their field or the evolutionary biologist who has made their way into the cancer lab. As a member of the former camp but now nearly more evolutionary biologist than cancer biologist I have written this introduction to the first camp. A large reason for the rapidly spreading employment of evolutionary theory in cancer biology is its seamless integration. Studying cancer as an evolutionary system is like reading the Old Testament in Hebrew, and so much of early cancer biology was lost in translation.

Lastly, although we have made quite the pass that *of course* cancer biology only makes sense as an evolutionary process we should recall here that virtually all of the progress made in treating cancers – including the occasional cure - have been done without evolution's invocation in the field. We should also be cognizant of the humbling, infrequent extensions to patients' lives we have so far been able to offer. Which leaves several important questions. What does evolutionary theory have to offer cancer biology? If cancer as an evolutionary

process is such a fundamental framework for understanding the disease, why has the emergence of the field of cancer evolution come so late? How can an understanding of cancer in non-human species translate into increased clinical knowledge? And perhaps most importantly, what hindrances still exist for a more broad integration of evolutionary theory with cancer biology? While many of these questions are addressed in the manuscripts that follow, the next decade will be flush with comprehensive solutions that we should be excited for. I finish this dissertation ever more convinced of the essential need for evolutionary theory in cancer biology and the promise of comparative oncology. There is grandeur in this view of life.

CHAPTER 2

A MISSING HALLMARK OF CANCER: DYSREGULATION OF DIFFERENTIATION

Chapter Introduction: Evolution All the Way Down

I am very fortunate in that much of the hard road ahead of applying evolutionary principles to cancer had already been paved for me by the time I began my doctoral work. Mutated somatic cells gaining a selective advantage over their healthy counterparts is a well-accepted evolutionary dynamic that defines carcinogenesis and disease progression. As is the selection pressure placed on tumors by cancer treatments that ultimately shape tumor resistance. However, given that the following manuscript was rejected by a prominent journal because “a cancer cell cannot have a fitness” I feel it is important to add some preceding clarification. As detailed in the general introduction, cancer evolution is not a special case or derived application of evolutionary theory. Such as are all scientific truths, cancers were commanded by Darwinian principles before they were described as such. And if we can agree that cancer is an evolutionary system then the very foundations of our modern understanding of cancer phenotypes, the hallmarks of cancer, must then be able to be described in an evolutionary context. To further this argument, given the evolutionary success of cancerous tumors compared to their surrounding tissue, an adaptationist lens is highly explanatory in defining cancer hallmarks.

When I first read Hanahan & Weinberg’s *The Hallmarks of Cancer* for the first time, their clarified simplicity introduced to me a very heterogeneous disease

that can yet be described by a discrete set of unifying phenotypes. The unifying logic of their seminal paper both assisted a 16 year old me in persevering through my first cancer research job and instilled in me a lasting appreciation for the cancer hallmarks. Still, as it was with Dmitri Mendeleev's first organization of the elements, few were aware that this list of phenotypes would continue to grow.

The duo themselves have since published a "next-generation" of cancer hallmarks which both added to the original list as well as invented a new category of pseudo hallmarks termed "enabling characteristics". Their 2011 paper argued that genome instability and tumor-promoting inflammation, although too transitory to be hallmarks, were so crucial to the successful acquisition of the hallmark phenotypes that they should be described adjacently. Now over a decade later Hanahan published yet a third hallmarks paper, which formally added the previous enabling characteristics as hallmarks and proposed an additional two; senescent cancer cells and phenotypic plasticity. Hanahan's publication in Cancer Discovery came after I received a lengthy rejection email from the editors at Nature Reviews Cancer, much of which emphasized the non-applicability of evolutionary principles in defining cancer hallmarks.

It was more than six months after Hanahan's publication that I, after continued struggles getting my own work through peer-review, took the concession of uploading my proposed hallmark onto a preprint server. Admittedly, the draft was well received but several individuals inquired on social media what I imagine many others were asking privately – is there, if any, difference between dysregulation of differentiation and phenotypic plasticity? In

Hallmarks of Cancer: New Dimensions Hanahan makes clear the strong relationship between phenotypic plasticity and lack of terminal differentiation, you cannot have one without the other. Which leaves what is the utilitarian difference in how each of the two proposed hallmarks are defined. Below I lay the foundation not only for why *specifically* dysregulated differentiation should be considered a hallmark but, perhaps more importantly, for how we should define hallmarks altogether.

Abstract

Cancer cells possess a nearly universal set of characteristics termed the hallmarks of cancer, including replicative immortality and resisting cell death. Dysregulated differentiation is present in virtually all cancers yet has not yet been described as a cancer hallmark. Like other hallmarks, dysregulated differentiation involves a breakdown of the cellular cooperation that typically makes multicellularity possible - in this case disrupting the division of labor among the cells of a body. At the time that the original hallmarks of cancer were described, it was not known that dysregulated differentiation was mechanistically distinct from growth inhibition, but now that this is known, it is a further reason to consider dysregulated differentiation a hallmark of cancer. Dysregulated differentiation also has clinical utility, as it forms the basis of pathological grading, predicts clinical outcomes, and is a viable target for therapies aimed at inducing differentiation. Here we argue that hallmarks of cancer should be near universal, mechanistically distinct, and have clinical utility for prognosis and/or therapy. Dysregulated differentiation meets all of these criteria.

Introduction

The identification of the hallmarks of cancer has been one of the most helpful and influential contributions to understanding cancer, because it brings simplicity, consistency and coherence to the otherwise overwhelming complexity of cancers(Hanahan & Weinberg, 2000, 2011). While cancer genomics has shown that each cancer is a unique mosaic of diverse genetic clones, evolutionary theory helps us understand why this diversity often converges on strikingly similar phenotypes represented by the hallmarks(Fortunato et al.,2017). We can view the hallmarks of cancer as the characteristics that are common across cancers, evolving consistently and independently in each cancer, because they confer a fitness benefit to the neoplastic cells over the surrounding normal somatic cells(Fortunato et al., 2017). All complex multicellular organisms require cooperation between their individual somatic cells(Aktipis C. Athena et al., 2015). Although complex multicellularity has evolved at least seven times(Knoll, 2011), there are five forms of cooperation upon which all multicellular organisms have converged: suppression of cell proliferation, controlled cell death, resource allocation, maintenance of the extracellular environment, and division of labor among the somatic cells(Aktipis C. Athena et al., 2015). Cancer, as a more general problem for multicellularity, can be understood as cells that cheat on the forms of cooperation necessary for building and maintaining a multicellular entity (Figure 1). All the current hallmarks of cancer map onto the five foundations of multicellularity, with one exception: there is no hallmark that corresponds to cheating on the division of labor among cells(Aktipis C. Athena et al., 2015). Here

we suggest that there should be an additional hallmark of cancer which corresponds to this breakdown in division of labor. A breakdown of division of labor among cells would manifest as cells not adopting the proper cell types that are necessary for the proper functioning of the organism, i.e., dysregulated differentiation.

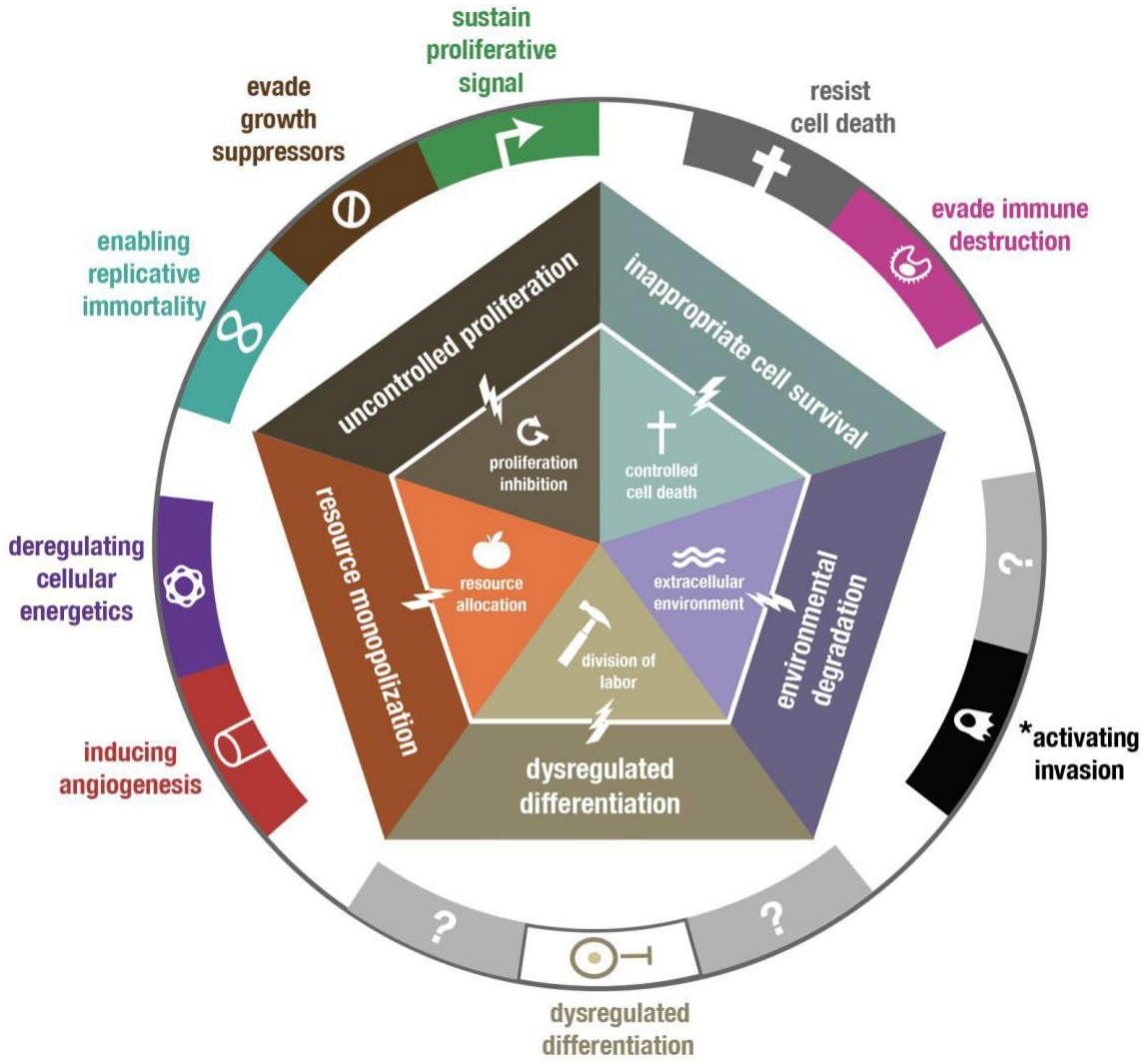

Figure 1. Cancer represents a breakdown of the foundations of multicellular cooperation that are necessary for multicellularity to succeed. The breakdown of every foundation of multicellularity corresponds to one or more of the existing hallmarks of cancer, with the exception of division of labor. Adding dysregulated differentiation as an additional hallmark of cancer fills this gap, corresponding to a breakdown in division of labor. There may well be other missing hallmarks,

represented here as other gaps in the periphery.

Not only does dysregulated differentiation fill this gap, it also is already a well-recognized universal feature of cancer that is mechanistically distinct from other hallmarks, important for prognosis and a promising target for therapy. As we will argue in this perspective, the cancer hallmarks should not only be universal across cancers, but they should also be mechanistically distinct from one another, as well as diagnostically and therapeutically useful (Table 1). By these criteria, dysregulation of differentiation should be considered a hallmark of cancer.

Cancer Hallmark	**Mechanistically Distinct**	**Diagnostically Functional**	**Therapeutically Relevant**
Sustained Proliferative Signaling	Constitutive activation of proliferative pathways (G. I. Evan & Vousden, 2001)	Proliferative markers such as Ki- 67 have been long used in staging/grading cancers (Gerdes, 1990)	Numerous compounds have demonstrated efficacy against known proliferative pathways (Feitelson et al., 2015)
Evade Growth Suppressors	Tumor suppressor pathways cannot be fully functional in metastatic disease (Amin et al., 2015)	Although characterized in childhood retinoblastoma, the RB pathway is mutated the majority of human cancers (Du & Searle, 2009)	RB mutation status can significantly guide the clinical management of a variety of cancer types (Du & Searle, 2009)

Avoid Immune Destruction	Through the expression of self antigens and manipulation via the tumor microenvironment, many tumor cells escape immune destruction (Finn, 2012)	Intratumor leukocyte infiltration can be used as a prognostic index determining anti- tumor immune activity (Fridman et al., 2011)	There a several therapeutic targets such as PD-1, PD- L1, CTLA4, and Th1 that can potentially counter immune evasion (Ribas & Wolchok, 2018; Vinay et al., 2015)
Enable Replicative Immortality	Cancer cells are able to restore and maintain telomere functionality(Hahn & Meyerson, 2001)	Telomerase activity provides insight to tumor differentiation status(N. W. Kim, 1997)	Targeting cyclin dependent kinases such as PI3K could trigger cancer cell senescence(Yaswe n et al., 2015). Telomerase is a target for cancer therapy(H.-S. Lee et al., 2018)
Activate Invasion & Metastasis	The ability of cancer cells to penetrate the basement membrane and disseminate into different tissues(Gupta & Massagué, 2006; Pachmayr et al., 2017)	Circulating tumor cells (CTCs) can be assayed for early detection of metastatic disease(Maheswar an & Haber, 2010)	Cell adhesion pathways can be targeted for therapy(D.-M. Li & Feng, 2011)
Induce Angiogenesis	Tumors cannot grow beyond 1-2mm^3 without establishing their own vasculature(Carmeli et & Jain, 2000)	Density of tumor supporting vasculature a useful prognostic tool(Weidner, 1995)	Anti-angiogenesis therapies target tumor resource delivery(Cherringto n et al., 2000)

Resist Cell Death	Disruption in the Bcl-2 signaling pathway precludes apoptotic response to DNA damage (Adams & Cory, 2007)	Determining the activity of BLC2 family proteins can reveal cells' ability to resist apoptosis (Glinsky & Glinsky, 1996; Letai, 2008)	Targeting death receptor ligands can trigger tumor cell death (Fisher, 1994; Kelley & Ashkenazi, 2004)
Deregulate Cellular Energetics	Cancer cells forgo oxidative phosphorylation, relying almost exclusively on glycolysis (R. A. Cairns et al., 2011)	Cancer cell metabolic phenotype predicts disease progression (Isidoro et al., 2005)	Recognized metabolic alterations are emerging as therapeutic targets (Teicher et al., 2012)
Dysregulated Differentiation	Tumor genomic profiles outline key genetic lesions that grant cancer cells their stem cell qualities (Ben-Porath et al., 2008)	Differentiation is the foundation of tumor grading (Elston & Ellis, 1991)	Differentiation therapy provides a unique therapeutic target with minimal toxicity (Nowak et al., 2009)

Table 1. Dysregulated differentiation, alongside all of the existing hallmarks, all meet our proposed criteria for hallmarks.

A universal feature of cancer

Dysregulation of differentiation is a universal feature of cancers (Hanahan, 2022; Tenen, 2003). Both genomic and histological evidence indicate that dysregulated differentiation is pervasive (Table 2). Cancers are generally diagnosed by histological features, detectable under a light microscope, that indicate that something has gone wrong in differentiation. Histological examination of differentiation status is a foundational method in the cancer grading system which has long been the cornerstone determining patient prognosis (Bansal et al., 2014; Bostwick, 1994). These histological aberrations of

differentiation are far ranging, including glands are that improperly formed or are missing altogether. Sometimes there is loss of regulation over a progenitor cell population, that has not fully differentiated, such that it expands to a pathological level, as occurs in most of the hematopoietic neoplasms (Tenen, 2003) as well as the undifferentiated clonal expansions in carcinomas. In fact, the generation of a new mass, a neoplasm, is probably impossible as long as differentiation is being properly regulated. Differentiation regulates the proper proportions and number of different cell types in every tissue. The epithelial-to-mesenchymal transition (EMT) common to many cancers is a further example of aberrant differentiation(L. Li & Li, 2015; H. Wang & Unternaehrer, 2019).

Cancer Type	Genomic Evidence of Dysregulated Differentiation	Histological Evidence of Dysregulated Differentiation
Breast	Down regulation of Gata-3 precludes healthy gland differentiation and disrupts luminal cell fate.(Asselin-Labat et al., 2007; Gawrzak et al., 2018; Kouros-Mehr et al., 2006, 2008)	Tumor differentiation status defines grading scale and strongly predicts patient prognosis(Elston, 1984; Elston & Ellis, 1991; Petushi et al., 2006)
Colorectal	NDRG2 is expressed at low or undetectable levels in high risk/poor prognosis colorectal adenomas(Lorentzen et al., 2007),(L. Shen et al., 2018)	Differentiation status of a tumor was more predictive of prognosis than invasive margin and DNA ploidy(Purdie & Piris, 2000)
Prostate	FOXA1 suppression in prostate carcinoma indicative of irregular differentiation patterns(Qin et al., 2012),(J. Kim et al., 2017),(W.-Y. Chen et al., 2019)	Lack of full differentiation in prostate cancer precludes the usefulness of serum prostate specific antigen in measuring tumor burden.(Bostwick, 1994; Partin et al., 1990)

Lung	TRPC channel disruption signals stemcell-like differentiation status(Hassan et al., 2009; Jiang et al., 2013; Lim et al., 2017)	Differentiation status is an independent predictor of prognosis in non-small cell lung cancer(Z. Sun et al., 2006; B.-Y. Wang et al., 2013)
Thyroid	Suppression of Notch signaling mediated differentiation (Ferretti et al., 2008; Somnay et al., 2017; Yu et al., 2013)	Diversity of thyroid carcinoma subtype founded largely on morphological differentiation(Akslen, 1993; Akslen & LiVolsi, 2000; Shaha et al., 1996)
Bladder	Renewal of Hedgehog signaling pathway can illicit differentiation factors that improve prognosis (Shin et al., 2014; Warrick et al., 2019)	Tumor cells reveal morphological indications of dysegulated differentiation before chromosomal aberrations(Pauwels et al., 1988; Wasco et al., 2007)
Stomach	Amplification of Notch1 intracellular domain maintains population of undifferentiated or poorly differentiated cells in carcinoma of the stomach(Choe et al., 1997; S. Hu et al., 2018; Katz et al., 2005)	Even well differentiated gastric carcinoma show histological evidence of disruption(Adachi et al., 2000)
Cervical	Expression of FOXC2 in cervical tissue correlates with increases in number of poorly differentiated cells(J. Wang & Yue, 2017),(X. Wu et al., 2019)	Disruption of healthy differentiation can be detected with light microscope and/or positron emission tomography(Kidd et al., 2009)
Non-hodgkin Lymphoma	Expression profiles show T-cell differentiation in B-NHL is skewed towards early stages (Anichini et al., 2006)	Phenotypic classification of tumor cells by degree of differentiation informs prognosis(Habeshaw et al., 1979; Seegmiller et al., 2007)

Endometrial	Karyotypic aberration patterns correlate with histological differentiation(Micci et al., 2004)	Tissue specific differentiation and hormone receptor positivity are key prognostic factors(Creasman, 1993; Mo et al., 2016; Tafe et al., 2010)
Leukemia	Pax5 loss and t(15;17) translocations both cause differentiation blocks in leukemias(G. J. Liu et al., 2014)	A review of differentiation therapy for leukemia(E. J. Lee et al., 1987; Nowak et al., 2009) Undifferentiated leukemia by light microscopy with myeloid features(E. J. Lee et al., 1987; B. U. Mueller et al., 2006; Nowak et al., 2009)
Kidney	Positive correlation between low PTEN expression and poorer differentiation(Que et al., 2018)	Differentiation level by subtype predicts patient outcome(Leibovich et al., 2010; Prasad et al., 2006)
Melanoma of the skin	Melanoma differentiation associated gene-7 (MDA7) expression is downregulated in advanced melanoma and virtually undetectable in metastatic disease(Ekmekcioglu et al., 2001)	Differentiation status and like- ness with other skin markings provides a baseline understanding of disease state
Lip, oral cavity	Absence of epithelial keratins defines a de-differentiated state in oral carcinomas(Leung et al., 2009; Ogden et al., 1993)	Morphological differentiation status, although particularly subjective in the oral cavity, still associated with patient outcome(Strieder et al., 2017; Warnakulasuriya, 2001)
Brain and Central Nervous System	Reactivation of Wnt signaling induce neural differentiation and cancer cell death (Boso et al., 2019; Guichet et al., 2013; Rampazzo et al., 2013; Q. B. Zhang et al., 2006)	Glioblastoma stem-like cells can hijack differentiation pathways to recruit vascularization(Ricci-Vitiani et al., 2008, 2010)

Ovary	Notch1 overexpression increases with decreasing extent of fully differentiated cells(McAuliffe et al., 2012; Rose, 2009; Rose et al., 2010; M. Wang et al., 2010)	Extent of morphologically poorly differentiated cells within ovarian tumor predicts prognosis(Malpica, 2008; Silverberg, 2000; Tafe et al., 2010)
Liver	MYC inactivation in an animal model of HCC induced differentiation and sustained regression of the tumor(Shachaf et al., 2004), Increased LEF1 expression in hepatocellular cancer is associated with poor cellular differentiation and worse prognosis, and regulates tumor differentiation through activation of NOTCH signaling pathways(Fang et al., 2019)	Well differentiated hepatocellular carcinoma presents atypically and yet retains histological evidence of differentiation abrogations(Calderaro et al., 2019; Jang et al., 2007)
Esophagus	22% of esophageal squamous cell carcinomas have mutations in genes that regulate esophageal squamous cell differentiation (NOTCH1, NOTCH2 or NOTCH3) (Gao et al., 2014) In squamous cell carcinoma, Notch3 is repressed by TGF*B*, which blocks terminal differentiation and leads to Notch1 mediated EMT (Natsuizaka et al., 2017)	Majority of esophageal carcinoma shows moderate to completely undifferentiated cell morphology(Trivers et al., 2008)
Larynx	Cyclin E overexpression in a majority of laryngeal carcinomas is a key driver of poorly differentiated tumors(Nadal & Cardesa, 2003).	Lymphoepithelioma is an undifferentiated carcinoma of the nasopharyngeal type with propensity for metastasis (Passler et al., 1999; Sarioglu et al., 2016; Stanley et al., 1985)

Multiple myeloma	Maintained B cell expression of CD38 perpetuates a sub-differentiated population of cells clonally related to the multiple myeloma plasma cells(Billadeau et al., 1993; Matsui et al., 2008)	Morphological indications of plasma cell differentiation level significantly predict clinical outcome(Bartl et al., 1987; Subramanian et al., 2009)

Table 2. Evidence for disruption of differentiation, both genetic and histopathological, in the 20 most prevalent types of cancers worldwide(*Cancer Today*, n.d.).

Convergent somatic evolution

Cells that stop devoting resources to the tasks inherent to that of their normal differentiated state, and instead devote those resources to proliferation and survival, will have a fitness advantage over cells that continue to devote resources to the specific tasks of their tissue type. Dysregulation of differentiation evolves independently in each cancer because it provides a selective advantage to those cells.

Differentiation is beneficial for organisms because it not only allows for the division of cellular labor, but also because it can lower cancer risk through reducing ongoing cell proliferation(X. Zhang et al., 2013). This appears to be one of the mechanisms that organisms have evolved to prevent somatic mutations and the expansion of clones that acquire selective advantages from those mutations(J. Cairns, 1975). In fact, there are many features of differentiated tissue architecture that function to constrain would-be clonal expansions. In intestinal crypts, which have a high rate of cell turnover, this function is performed by basal apical polarity axis maintained through a basement membrane attachment requirement, apical tight junctions between adjacent cells, and basal hemidesmosomal attachment complexeses (Chandramouly et al., 2007; Clevers, 2013; Gehart & Clevers, 2019; Snippert et al., 2010; van der Heijden & Vermeulen, 2019). Consequently, neoplastic cells gain a cell-level fitness advantage by evading those constraints(Fortunato et al., 2017). The suspension of proliferative abilities in fully differentiated cells is one of the major mechanisms of somatic-level evolutionary suppression, in other words it is a

cancer resistance mechanism. However, this also means that there is strong selective pressure on neoplastic cells to evolve the ability to evade full differentiation.

Cairns first pointed out in 1975 that if mutations are gained in transit amplifying cells, which are only partially differentiated, these cells will quickly be flushed from the body with little chance to accumulate additional mutations necessary to cause cancer(J. Cairns, 1975) (Figure 2). In this way, differentiation in tissues with high cell turnover acts as a tumor suppressor. Follow-up mathematical and computational models have shown that alterations in differentiation are likely some of the most universal early lesions in neoplastic progression(Haeno et al., 2009; Sprouffske et al., 2011).

Both stem cells and transit amplifying cells gain fitness advantages from disrupting differentiation(Sprouffske et al., 2013). However, there are generally many more transit amplifying cells than stem cells and so some mathematical models predict that most cancers derive from transit amplifying cells, even if that requires additional mutations to disrupt differentiation(Haeno et al., 2009). In order to become cancerous, transit amplifying cells must avoid the fate of being sloughed from the off of a proliferating tissue (Figure 2b). Any stem cell that disrupts differentiation and divides symmetrically, producing two daughter stem cells, will have a fitness advantage over other stem cells that divide asymmetrically and use some of their resources to produce non-stem cells (Figure 2a). In summary, there are good evolutionary reasons to expect that virtually all cancer cells can gain a fitness benefit from disrupting differentiation,

which explains why dysregulation of differentiation consistently evolves and is a universal feature of cancers.

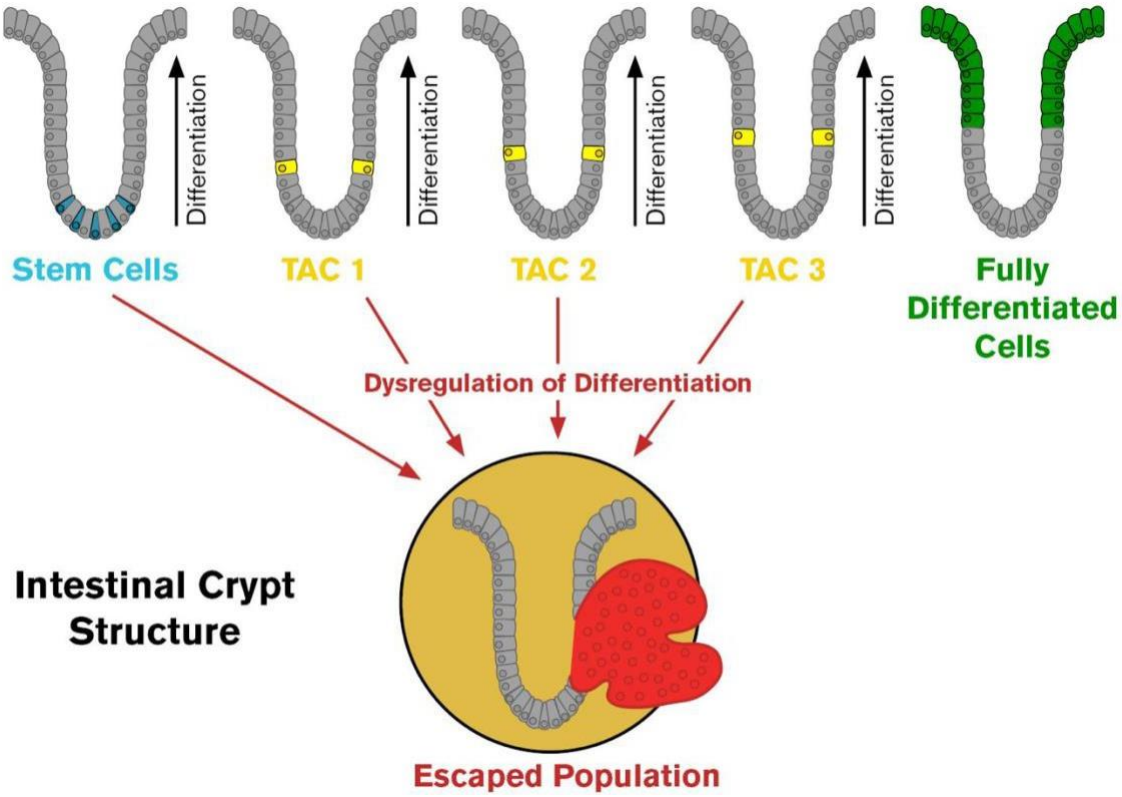

Figure 2. In order for a neoplasm to grow, cells must somehow evade the inexorable conveyor belt of differentiation that transforms stem cells into progressive stages of transit amplifying cells, eventually becoming fully differentiated cells, and finally exiting the tissue by apoptosis. There are two ways a neoplasm may form: **a.)** A clone of stem cells may stop producing transit amplifying cells, only dividing symmetrically to produce daughter stem cells. That clone will have a competitive advantage over any stem cell clones that continue to use some of their resources to produce transit amplifying cells. This may be due to an abrogation in the clone's differentiation pathways or through the gaining of independence from stem cell niche signals that would otherwise be required to maintain the stem cell state. **b.)** Alternatively, transit amplifying cells may stop differentiation and so effectively step off of the conveyor belt of differentiation. This gives the non-differentiating (and thus self-renewing) transit amplifying cells a competitive advantage over transit amplifying cells that continue to differentiate.

Mechanisms of dysregulation

In their original hallmarks paper, Hanahan and Weinberg discuss the apparent strategy of tumor cells to promote growth by avoiding terminal differentiation. The authors specifically cite the Mad-Max complex, the inactivation of APC/B-catenin pathway in colon carcinogenesis, and the erbA oncogene in avian erythroblastosis(Hanahan & Weinberg, 2000). At the time, the dysregulation of differentiation was not included in the hallmarks of cancer because the mechanisms of differentiation could not be distinguished from an insensitivity to antigrowth signals and limitless replicative potential. It was not clear whether loss of differentiation was simply a loss of growth inhibition or an independent factor in carcinogenesis.

In general, differentiation and growth inhibition are tightly, and mechanistically coordinated. However, there are instructive cases of fully differentiated cells that are still proliferative, including beta cells in the pancreas, hepatocytes in the liver, T-cells, and fibroblasts in numerous tissue types(Grotendorst et al., 2004; W.-H. Liu et al., 2010; Luckheeram et al., 2012; Manohar & Lagasse, 2014; Min, 2018; Visco et al., 2009; Zhong & Jiang, 2019). These exceptions show that there is a fundamental distinction between loss of proliferative ability and differentiation, though they co-occur often.

There has been ample documentation of interruptions in key differentiation pathways, separate from growth inhibition pathways, that are conserved across cancer types, ultimately preventing true terminal differentiation. Notch signaling plays a complex, and not fully understood, role in distinct differentiation signaling

pathways(Sriuranpong et al., 2001). In T-cell acute lymphoblastic leukemia (T-ALL) chromosomal translocations result in constitutive *Notch1* signaling that precludes terminal differentiation(Cullion et al., 2009; Ferrando, 2009; O'Neil et al., 2006; Sjölund et al., 2005; Sulis et al., 2008; Vilimas et al., 2007; Weng et al., 2004). Rangarajan and colleagues demonstrated that *Notch1* deletion in keratinocytes resulted in hyperplasia and generalized dysregulation of known differentiation markers(Rangarajan et al., 2001). In addition, constitutive expression of the MYC oncogene is common in human cancers and has a well-established role in prevention of differentiation and sustaining proliferative signals(Cole, 1986; Coppola & Cole, 1986; Dmitrovsky et al., 1986; Freytag, 1988; Pelengaris & Khan, 2003; Prochownik & Kukowska, 1986; C. Sun et al., 2008; Wilson et al., 2004). For instance, in a murine model of liver cancer inactivation of MYC was sufficient to differentiate the tumor into normal hepatocytes(Shachaf et al., 2004). Interestingly, c-myc expression drives the differentiation of keratinocytes where *Notch1* appears to play the role of a tumor suppressor(Gandarillas & Watt, 1997; Klinakis et al., 2011; Panelos & Massi, 2009; Watt et al., 2008).

The identification of differentiation specific pathway alterations holds the potential to serve as an indicator for therapeutic response. In the crypt structures of the intestinal epithelium, progenitor stem cells are characterized by high expression of Leucine-rich repeat-containing G-protein coupled receptor (LGR5)(He et al., 2014). Similarly high levels of expression are seen in colorectal cancers, where it is indicative of catastrophic Wnt/β-catenin signaling

deregulation and worse patient outcomes(He et al., 2014; Herbst et al., 2014; X.-S. Wu et al., 2012).

Well-differentiated tumors

Well-differentiated cancers sometimes can be difficult to distinguish from reactive changes in tissue, such as hyperplasia, or benign tumors by light microscopy. However, even if indiscernible by visual inspection, molecular evidence shows the presence of dysregulated differentiation in well-differentiated tumors. Well-differentiated tumors exhibit a less differentiated molecular profile with elevated expression of precursor genes and lower expression of tissue specific genes compared to healthy tissue(Enane et al., 2017). Gene expression studies in histologically differentiated thyroid cancers have found a disruption in differentiation on a molecular level as compared to benign thyroid tissue. Using primary thyroid cancers, Yu *et al* demonstrated that Notch-1 expression was downregulated in differentiated thyroid cancer tissues compared to benign thyroid tissues and that decreased Notch-1 expression was associated with more aggressive tumors with extrathyroidal invasion(Yu et al., 2016). Restoration of Notch-1 expression in a metastatic, differentiated thyroid carcinoma cell line led to a reduction in cell growth and tumor cell migration(Yu et al., 2016). The Cancer Genome Atlas Research Network investigated the relationship between driver mutations BRAFV600E and RAS and differentiation in papillary thyroid cancer, a typically well-differentiated cancer by histology(Cancer Genome Atlas Research Network, 2014). Differentiation of over 350 PTCs was quantified and

scored by measuring mRNA expression of 16 thyroid function genes, with a lower score indicating decreased differentiation(Cancer Genome Atlas Research Network, 2014). Interestingly, increased differentiation scores were correlated with the PTC driver mutation BRAFV600E, while decreased differentiation scores were correlated with the driver mutation RAS(Cancer Genome Atlas Research Network, 2014). Further, upon pathological examination, tumors with lower differentiation scores by mRNA expression were found to have subtle architectural changes that generated more poorly formed and complex papillary structure with fewer follicles(Cancer Genome Atlas Research Network, 2014). These findings suggested that certain driver mutations may contribute to decreased differentiation in thyroid cancer(Cancer Genome Atlas Research Network, 2014). As the new molecular tools under development are advanced for clinical application, the feasibility of identifying lack of terminal differentiation even in the most well-differentiated cancers increases.

Prognostic importance

Poorly differentiated cancer cells are known to be much more aggressive than their well differentiated counterparts, a fact which plays a critical role in predicting patient outcome(Adachi et al., 2000; Bostwick, 1994; Busto Catañón et al., 2001; Nishida et al., 1999). Well-trained pathologists have long been able to accurately assign patient prognosis through tumor grade although the process is heavily burdened with the inherent subjectivity in assessing differentiation optically and the morphological variation that is inherent in most tumors. Advances in cancer genomics have validated genetic attributes that resemble

stem cells(Schwede et al., 2013; Smith et al., 2015; Toraih et al., 2016) as prognostic indicators. Similar to histopathological grading systems, differentiation gene-expression profiles can predict patient outcomes(Schwede et al., 2013; Smith et al., 2015; Toraih et al., 2016). A 2017 study examined the global gene expression profile of cancer cells and stratified them based on their distance in expression from that of stem cells to fully differentiated cells, using several different histologies including carcinomas, sarcomas, and hematologic malignancies(Riester et al., 2017). This methodology allowed for the derivation of a novel cancer gene expression signature found in all undifferentiated forms of the diverse cancers studied. For all subtypes analyzed, tumors most similar in expression to stem cells were both histologically less differentiated and clinically more aggressive. Furthermore, they also demonstrated that where a cancer fell on this spectrum predicted the patient's survival. Work by Riester et al.(Riester et al., 2017)and others has shown that there are objective measures of cellular differentiation, utilizing descriptive genetic profiles that detail where on a spectrum from "stemness" to full differentiation a given cancer cell lies. Grading with molecular assays that measure the hallmarks of cancer enriches our ability to make clinical predictions while introducing novel quantification of differentiation status through genetic analysis.

Promising clinical opportunities

Differentiation should be considered a hallmark of cancer not only due to its universality and distinct cellular mechanisms that drive cancer, but also because the biological mechanisms can be targeted by available therapies that

have already shown promise in the clinic. Rather than killing both healthy and tumor cells as do typical chemotherapeutics, differentiation therapies capitalize on the ability of cytokines to promote terminal differentiation of tumor cells and halt their capacity to self-renew (Dow et al., 2015; Mueller et al., 1998; Pham et al., 2011; Sarraf et al., 1998). This option is especially promising for patients suffering from comorbidities who are unable to receive high-dose chemotherapy due to its significant toxicity.

The first successful clinical application of differentiation therapy was the use of All-*trans* Retinoic Acid (ATRA) for acute promyelocytic leukemia (APL). ATRA induces APL blasts to terminally differentiate(Nowak et al., 2009). The current standard of care for treatment of APL involves the combination of ATRA and arsenic, making APL now a highly curable disease with 5-year disease-free survival rates that exceed 90%(Z.-Y. Wang & Chen, 2008).

Outside of APL, differentiation therapy has been gaining traction in the treatment of acute myeloid leukemia (AML)(Christian et al., 2019; Ferrara et al., 2001; Johnson & Redner, 2015; Nowak et al., 2009; Petrie et al., 2009). A recent preclinical study has identified a novel, highly potent and selective inhibitor that induces differentiation *in vitro* and *in vivo* by inhibiting dihydroorotate dehydrogenase across multiple AML subtypes(Christian et al., 2019). A promising phase-1 trial of this inhibitor, BAY 2402234, is currently ongoing for myeloid malignancies (NCT 03404726).

Whether differentiation therapy shows similar effects in cancers apart from

the hematological malignancies is worth investigating. Cancer stem cells (CSCs), also known as tumor-initiating cells, represent one such target for differentiation therapy(Frank et al., 2010; Y. Hu & Fu, 2012; Massard et al., 2006; Sell, 2004, 2006; Takebe et al., 2011; Takebe & Ivy, 2010). First identified in AML in 1997, they have since been identified in brain cancer, colon cancer, pancreatic cancer, prostate cancer, melanoma, and more(Lapidot et al., 1994; Visvader & Lindeman, 2008). They are highly resistant to traditional chemotherapy and radiotherapy, which may be due in part to their relative slow growth and high expression of anti-apoptotic proteins(Todaro et al., 2007). Differentiation therapy is a promising tactic that may induce these CSCs into non-stem cancer cells with limited self-renewal potential. These non-stem cells could possibly be better targeted by conventional therapies(Lombardo et al., 2011; Yan et al., 2016).

Another logical application of differentiation therapy would be in tumors that are collectively known as “blastomas” or small round blue cell tumors, named after their histological appearance, which is monotonous and characterized by lack of differentiation features. These relatively undifferentiated tumors originate from stem cell progenitors and occur almost exclusively in pediatric patients. Neuroblastoma is one of the small round blue cells tumors. It is famous for frequent spontaneous regression or differentiation into a benign ganglioneuroma(Brodeur, 2018). Recent evidence shows that neuroblastomas are composed of cells from two super-enhancer associated differentiation states: undifferentiated mesenchymal cells and committed adrenergic cells(van Groningen et al., 2017). Nevertheless, cells from either state can interconvert,

highlighting a potential mechanism of tumor relapse as mesenchymal cells are known to be relatively resistant to chemotherapy(van Groningen et al., 2017). Furthermore, these preserved differentiation pathways have been successfully targeted in vitro with retinoids(Reynolds, 2000; Reynolds et al., 2003), a response categorized with cell proliferation arrest and a markedly lower MYCN expression(Reynolds et al., 2003).

Differentiation therapy can only work if some differentiation pathways remain intact in a cancer and can be stimulated by an intervention. Due to natural selection at the somatic level for the dysregulation of differentiation, it may not always be possible to induce differentiation**.**

Conclusions

Dysregulation of differentiation is a universal phenotype, found in virtually all cancers (Table 2). The degree of differentiation has long been used in oncology for diagnosis as well as prognosis, and advances in genomic analyses have shown promise for improving prognosis. Dysregulation of differentiation is molecularly distinct from the other hallmarks, including evading growth suppressors, and it has been successfully targeted for therapy in acute promyelocytic leukemia and neuroblastoma. Further, it is clear that dysregulated differentiation is a breakdown of multicellular cooperation, and the only aspect of this breakdown of multicellular cooperation that is not already represented in the hallmarks of cancer(Aktipis C. Athena et al., 2015). Together, this suggests that dysregulated differentiation is a missing hallmark that should be added to the

commonly accepted list of shared phenotypes of cancer.

Acknowledgments

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CHAPTER 3

CANCER ACROSS VERTEBRATES

Chapter Introduction: The Forest and the Trees

Glancing at **Figure 1** in the *Cancer Across Vertebrates* manuscript the reader may catch that I introduce a paradox of my own. If some fundamental vulnerability to cancer is at the very core of metazoan life how could so many species be highlighted with zero observed neoplasms? Although I am fully confident in the validity of the zoological data collected and curated for these projects, I do not believe them to be above a reasonable standard measurement error. I have no doubt that all the species used in these studies, and those like it, are vulnerable to cancer. It matters very little if the zero observed neoplasms for any given species is truly zero. When comparing the Rodrigues fruit bat (*Pteropus rodricensis*) and the ferret (*Mustela putorius*) I am much more confident in the distance between their respective cancer prevalence than the actual values of said prevalence. In another way, regardless of if the fruit bat has truly 0% cancer prevalence and the ferret has 70% or the fruit bat has 10% and the ferret has 80%. I have been burdened firsthand with the arguments over zoo vs. wildlife data and what methodologies are most appropriate for identifying tumor specimens. There is a substantial, perhaps unconquerable, gap between perfect quality cross-species cancer data and data of sufficient quality where the patterns between data points reflect reality. It has not been the point of this study, nor should it be for anyone's, to prove the precise cancer prevalence for any specific species. My aim is to account for and make sense of the evolution that

explains the gaps between any two species' cancer risk.

Collecting and curating such a database as the one these comparative oncology studies are predicated on may have been the biggest triumph. I have gone to considerable length in the *Cancer Across Vertebrates* paper to address the many rational objections to working with zoo data. More importantly, I have also several analyses that point to many of the more common objections being unfounded. The artificial extension of lifespan afforded to species under human care due to the removal of predation and the access to veterinarian healthcare is often pointed to as a likely cause of inflated cancer prevalence. **Figure 5** in the main text of the article highlights the proportion of tumors (both neoplasms and malignancies) that occurred within the natural lifespan of the species. It overwhelmingly demonstrates that extension of lifespan does not explain most tumors in our database. The objection is logically deduced from the fact that age is the single biggest risk factor for developing cancer. Even though we know quantifiably this does not translate to an inflation in cancer risk in our database there is another, theoretical, defense that I am equally satisfied with. Let us suppose something absurd, that all the tumors found in our database are the result of an artificially protracted lifespan in zoo animals. We would still be able to identify patterns of differences in cancer risk across species and come to similar conclusions. For me, the age-specific analysis only further validates our database and allows us to rely much less on future studies to validate what we have found in wild species. A final note on the issue with life extension for animals under human care. This assumed extension of life, although certainly

true in some species, is not shared equally across all species commonly held in zoos. There are many species that seem much less successful in zoos than in the wild and therefore we can only say with confidence that those species that are largely prey animals in the wild uniformly enjoy such an extension of life. We can transition here to make another clarification that I was previously unaware that I had to make. Unbeknownst to me there is some contention on how tumor prevalence should be calculated. A common complaint is that only one or a few individuals within a species that have some individual or environmentally specific increase in cancer prevalence may inflate the measured cancer prevalence for the entire species surveyed. Several methodologies have suggested for calculated species-level cancer prevalence but for these analyses here one individual may only be counted once. Should any individual animal within a species have one tumor or a dozen, they only add one data point to their species' cancer prevalence.

Comparative Phylogenetics in Comparative Oncology

Another response to the point on the accuracy of zoo cancer data was made with a modification to R package "phytools" developer Liam Revell's pgls.SEy(Phylogenetic Generalized Least Square Standard Error of Y) function. I was a student of Liam Revells and I owe him a great deal for inspiring me to apply such a broad swath of phylogenetic comparative methods to cross-species cancer data. So, given that we can assume some standard error of measurement in our species' level cancer data, it seemed fitting to use his pgls function that accounts for a measurement of such error. The difficulty I encountered with his

pgls.SEywas that while it worked in the way intended, I could not retrieve a maximum likelihood lambda parameter from it. Pagel's lambda, which is the proportion of the variance in the trait value that is explained by phylogenetic distance (i.e. evolutionary relatedness), has been of significant interest to me throughout this work. When analyzing a trait through these methods for the first time I felt it inappropriate to assume the lambda parameter. To be satisfied with my analyses for this paper I wanted to both account for a standard error of measurement (here we simply used 1/√Total Individuals) and measure the lambda parameter. Further, I did not use the normal standard error of a binomial proportion due to the additional weight the lower values would add to the regression. The function written for this paper, and included in the supplementary, pglsSEy.Pagel, simply builds upon his pgls.SEyfunction but adds the optimization to measure lambda.

Phylogenetic comparative methods are overwhelmed with counter arguments on what defines best practices, which parameters on trait evolution are the best to measure, and which parameters serve as the red herrings. Perhaps no parameter is more contested than Pagel's lambda. But as we begin to see the era of these methods applied to traits relevant broadly to evolutionary medicine, I will offer a small defense for the coefficient in comparative phylogenetics I find most interesting, and how it led me to pursue analyses in comparative oncology that I otherwise would not have.

Equally excited and hesitant, we report in *Cancer Across Vertebrates* that a non-neutral model of trait evolution, Ornstein-Uhlenbeck, was the best fitting

model to explain the diversity of cancer susceptibility within each of the three clades we analyzed. Most models of continuous trait evolution involve the addition of parameters scaffolded to the earliest model of trait evolution, brownian motion. A brownian motion model of trait evolution reflects stochastic changes in the ancestral trait value where the variance in trait values, across species, increases linearly with time. Brownian motion evolution on a phylogeny is an example of a random walk process, since change in the trait value between any two species is random in both direction and distance. It is defined by two parameters, the mean trait value and the rate of evolution (σ^2). The product of these parameters, $\sigma^2 \times$ *time (t)*, is equal to the variance in the resulting trait values.

Three principle factors can drive the change in trait values from generation to generation; mutational burden, genetic drift, and the selection for a trait towards some optimum (adaptation). A central pursuit of comparative phylogenetics has been to determine which of these processes is most explanatory for a given trait and a given phylogenetic space. Ornstein-Uhlenbeck adds an additional parameter to the Brownian motion model, alpha (α), which is a measurement of the tendency for the trait value to return a set optima. The higher the α parameter, the greater the strength of stabilization around a mean trait value. Although often interpreted as a model of stabilizing selection, the stabilizing force portrayed in the model has some important distinctions with the population genetics usage of stabilizing selection. The stabilization of the trait value in Ornstein-Uhlenbeck is describing the constraint of trait evolution based

on the optimal value rather than describing the mean trait value's position on a fitness landscape. Simply, based on the variation in the trait value across the phylogeny there is an implicit assumption about its optimality, and that assumption is based outside the context of an explicit fitness landscape.

In the context of cancer evolution, the significance of the Ornstein-Uhlenbeck model fits well within the life history framework of cancer risk. Given the background ecology for a given species, an overinvestment in cancer suppression when it is inappropriate (fast life history species) is just as costly as an underinvestment when it is needed (slow life history species). Deviations in investment in somatic maintenance that occur spontaneously due to genetic drift and mutation should be sharply curtailed in the population by natural selection.

Abstract

Cancer is pervasive across multicellular species. Are there any patterns that can explain differences in cancer prevalence across species? Using 17,536 necropsy records for 327 species spanning three clades (amphibians, sauropsids and mammals) we found that neoplasia and malignancy prevalence increases with adult weight and decreases with gestation time, contrary to Peto's Paradox. Evolution of cancer susceptibility appears to have undergone sudden shifts followed by stabilizing selection. Outliers for neoplasia prevalence include the common porpoise (<1.3%), the Rodrigues fruit bat (<1.6%) the black-footed penguin (<0.4%), ferrets (63%) and opossums (35%). Discovering why some species have particularly high or low levels of cancer may lead to a better understanding of cancer syndromes and novel strategies for the management

and prevention of cancer

Introduction

Cancer is a ubiquitous problem for multicellular species (Aktipis C. Athena et al., 2015) and a leading cause of death in humans (Ahmad & Anderson, 2021). Every multicellular body is a cooperative cellular system, with cells suppressing replication (Pepper et al., 2007), dividing labor (Kirk, 2005), sharing resources (Nedelcu, 2020), regulating cell death (G. Evan & Littlewood, 1998) and taking care of the extracellular environment (Aktipis C. Athena et al., 2015). However, cooperative systems are susceptible to cheaters, which emerge as cancers in multicellular organisms (A. Aktipis, 2020). Because cancer cells can outcompete normal cells with respect to replication, survival, resource use and other cellular behaviors, natural selection within the body can favor cancer cells via somatic evolution.

Cancer has been a strong selective pressure on multicellular organisms and mechanisms for cancer suppression likely co-evolved along with the evolution of multicellularity (DeGregori, 2011; Domazet-Loso & Tautz, 2010). Despite this persistent selective pressure of cancer, species vary in their investment in cancer defenses across the tree of life. Sir Richard Peto predicted in 1977 that the risk of cancer should scale with the number of cells in an organism and the length of its lifespan (Peto, 1977). This prediction is based on the fact that tumors evolve from single cells, partially due to the accumulation of somatic mutations over time (Peto, 1977). His observation that cancer risk does not appear to increase with increases in body mass and longevity across species

(Peto, 1977), a phenomenon known as ‘Peto’s paradox’, launched the field of comparative oncology (Nunney et al., 2015).

Early work in comparative oncology found that birds, and to a lesser extent reptiles, develop fewer neoplasms than mammals (Duke et al., 2022; Effron et al., 1977b; Kitsoulis et al., 2020). While single case studies have been reported (Madsen et al., 2017a), it has been difficult to estimate true neoplasia prevalence in these taxa. In 2015, we published neoplasia prevalence estimates in 37 mammals and reported support for Peto’s Paradox, that is, bigger, longer-lived species do not get more cancer (Abegglen et al., 2015). Follow up studies have supported Peto’s Paradox and demonstrated the ubiquity of cancer across mammals (Boddy et al., 2020b; Vincze et al., 2022). The extensive variation in cancer risk across vertebrates provides a unique opportunity to identify species with exceptional cancer resistance that can lead to new discoveries of cancer resistance mechanisms outside the traditional human and murine studies. Additionally, the discovery of cancer vulnerable species could lead to new insights into cancer syndromes as well as provide spontaneous ’natural’ animal models of disease that can help us gain a better understanding of various types of cancer and their treatments. Here we present a large, curated database of tetrapod veterinary necropsy records, including 17,536 individual animals across 327 species of animals, encompassing reptiles, birds, amphibians, and mammals. Because necropsies typically are diagnosed with “neoplasia” which includes both benign and malignant tumors, we developed a terminology dictionary to distinguish benign from malignant neoplasms in the necropsy

reports. We calculate and analyze both neoplasia prevalence as well as malignancy (cancer) prevalence. Only a subset of benign neoplasms evolve into cancers over a lifetime, so neoplasia prevalence is always greater than or equal to malignancy prevalence. We also tested for age bias in the animals that died with neoplasms or cancers.

Results

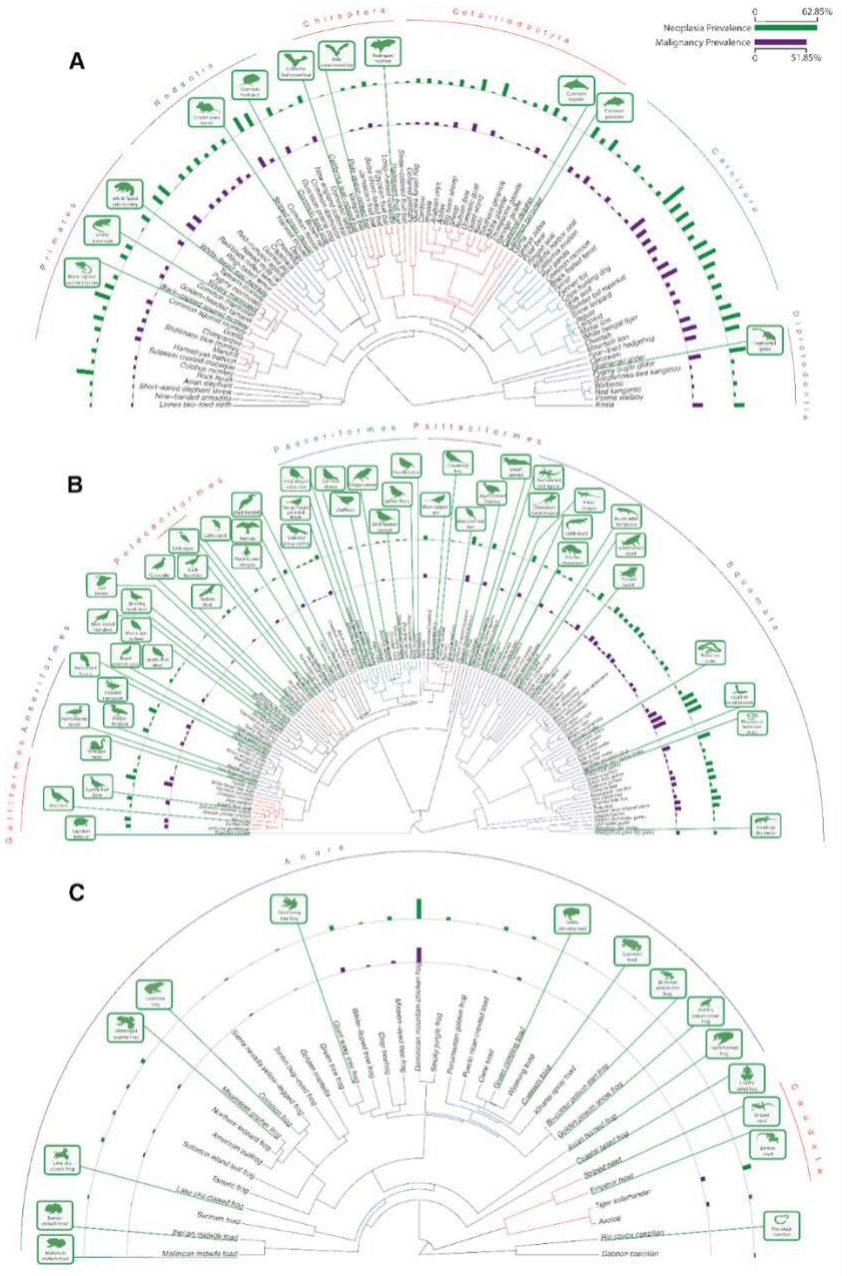

Figure 1. Neoplasia and malignancy prevalence across mammals (**A**), sauropsids (**B**), and amphibians (**C**). Silhouetted species indicated that zero neoplasms were reported.

Variation in Cancer Risk Across Clades

We found evidence of neoplastic disease in necropsies across all analyzed taxonomic clades (Fig.1). Mean prevalence of neoplasia (mean = 9.07%, range = 0% - 62.86%) and malignancy (mean=5.91%, range=0%–40.95%) at death was highest in mammals (mean: Neoplasia= 15.28%, Malignancy = 9.82%; range: Neoplasia = 0% - 62.85%, Malignancy = 0% - 40.95%), followed by sauropsids (mean: Neoplasia=6.40% Malignancy=4.33%; range: Neoplasia= 0% - 39.13%, Malignancy: 0% - 34.78%) and amphibians (mean: Neoplasia = 4.16%; range: Neoplasia = 0% - 45.83%, Malignancy = 0% - 33.33%; Fig. 2), which confirms previous studies (Effron et al., 1977b; Kitsoulis et al., 2020). Because reptiles are not a monophyletic clade, we have grouped them with birds in the sauropsida clade for the purposes of analysis. Despite a lower mean prevalence for both benign and malignant tumors, sauropsids and amphibians show a wide range of neoplastic disease burden across species. There is a small but highly statistically significant correlation between the prevalence of benign neoplasms and the prevalence of malignant neoplasms across species (r=0.2, p<0.0001, Fig. S64). Supplementary Tables 1 and 2 list the species with the highest and lowest neoplasia and malignancy prevalences, as well as the proportion of neoplasms that are malignant. Among the vertebrates with the highest prevalence of neoplasia (Suppl. Table 8), 63% of ferrets died with a neoplasm (45% of which was lymphoma), 56% of opposums died with a neoplasm (46% of which was in the lung), and 45% of hedgehogs died with a neoplasm (42% of which was in the alimentry tract).

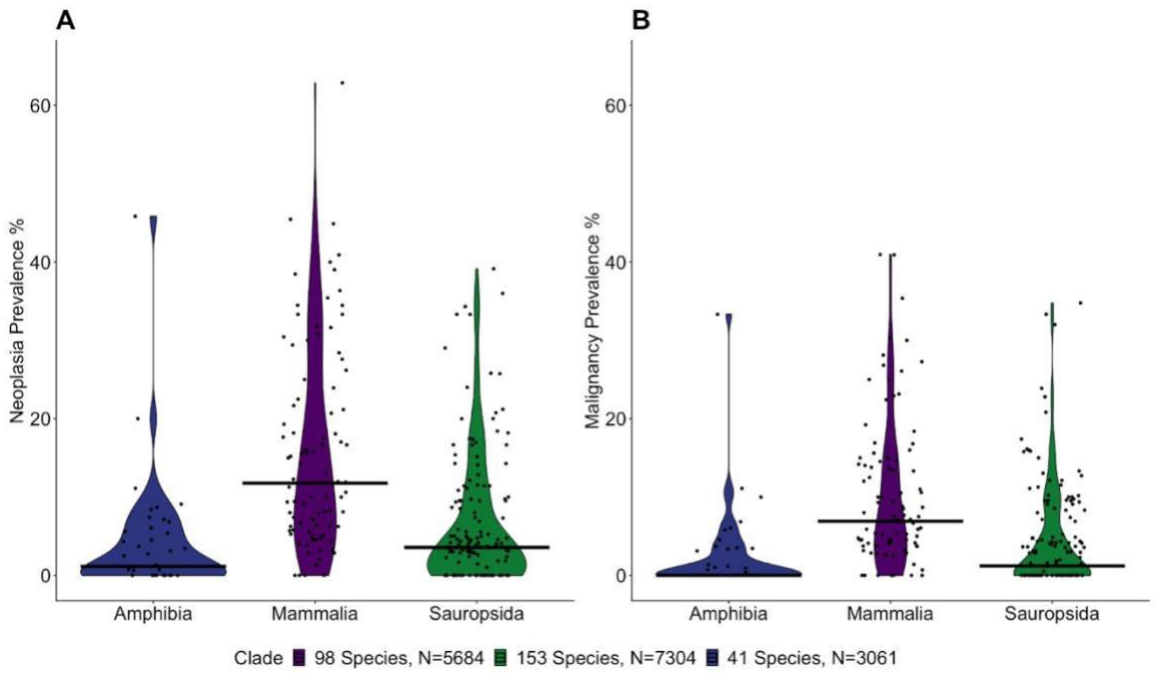

Figure 2. Distributions of of **A.** neoplasia (Kruskal-Wallace test: $p = 2.906 \times 10^{-12}$) and **B.** malignancy (Kruskal-Wallace test: $p = 6.519 \times 10^{-11}$) prevalences are different across three clades, Amphibia, Mammalia, and Sauropsida (Reptilia and Aves). Dots show the estimated species neoplasia prevalence and bars show the median for the clade. Neoplasia and malignancy prevalence for species were calculated by the proportion of the reported lesions among the total number of necropsies for that species.

Life History Analyses of Neoplasia Prevalence

Evolutionary life history theory provides a framework for understanding the tradeoffs governing species' survival and reproduction (Bielby et al., 2007; Lika & Kooijman, 2003). Life history theory can be used to explain how species level traits shape organismal cancer risk based on trade-offs between investment in somatic maintenance (e.g., cancer suppression) and reproduction or growth. Several smaller studies have shown that specific life history traits can serve as prognostic indicators of neoplasia prevalence in animals managed under human care (Boddy et al., 2020b; Kokko & Hochberg, 2015). We tested for relationships between life history factors and neoplasia or malignancy prevalence, controlling

for phylogenetic relatedness, and weighting species data points by the number of necropsies in our dataset. In contrast to previous studies (Abegglen et al., 2015; Boddy et al., 2020b; Vincze et al., 2022), we found an increase in neoplasia prevalence with increases in body mass (2.1% neoplasia per Log_{10}g, $p = 0.007$; 0.65% malignancy per Log_{10}g, $p = 0.287$) and maximum longevity (0.01% neoplasia per Log_{10}g, $p = 0.02$; 0.0047% malignancy per Log_{10}g, $p = 0.276$), not supporting Peto's Paradox (Fig. 3). Animals with longer gestation times also get fewer malignancies (-5.56% malignancies with Log_{10} months, $p = 0.02$; Fig. 3C).

A multivariate model containing all significant predictors of neoplasia or malignancy (adult weight, maximum longevity, and gestation time) shows that both adult body weight (2.9% neoplasia per Log_{10}g, $p = 0.01$) and gestation time (-18.6% neoplasia per month, $p = 0.0001$) provide independent information for estimating neoplasia prevalence. Because gestation time and adult weight are correlated ($r = 0.50$, $p = 2.2 \times 10^{-16}$), but have the opposite relationship to neoplasia and malignancy prevalence, we tested the two-variable model and found that when controlling for adult weight (3.8% neoplasia per Log_{10}g, $p = 0.0005$), gestation time is also a significant predictor of neoplasia prevalence (-15.8% neoplasia per Log_{10} months, $p = 0.001$; $R^2 = 0.27$), and vice versa. When controlling for gestation time, adult weight predicts malignancy prevalence, and when controlling for adult weight, gestation time also predicts malignancy prevalence (0.68% malignancies per Log_{10}g, $p = 0.0008$; -6.61% malignancies per month of gestation, $p = 0.0417$; $R^2 = 0.198$). Adult weight and gestation time were still statistically significant (adjusted $p<0.05$) predictors of neoplasia and

malignancy prevalence after a 10% false discovery rate correction for multiple testing.

We found no evidence of a relationship between litter or clutch size and neoplasia prevalence (Figs. S21, S22). However, when we restrict the analysis to mammals, litter size is positively associated with both neoplasia and malignancy prevalence (neoplasia: $p = 0.02$, $R^2=0.55$; malignancy: $p = 0.03$, $R^2= 0.2$; Suppl. Figs. 26 & 27), supporting our earlier analysis of 37 mammals from the San Diego Zoo (Boddy et al., 2020b). We also found that time to sexual maturity, growth rate and basal metabolic rates (which were only available for mammals) were not significant predictors of neoplasia or malignancy prevalence (Figs. S11, S12, S13, S14, S17, and S18). In addition to calculating the prevalence of neoplasms and malignancies, we also calculated the proportion of neoplasms that were malignant, which is a measure of the likelihood that a benign neoplasm transforms into a malignant one. We found no statistically significant relationships between any of those life history factors and the proportion of neoplasms that were malignant (Figs. S11, S13, and S17).

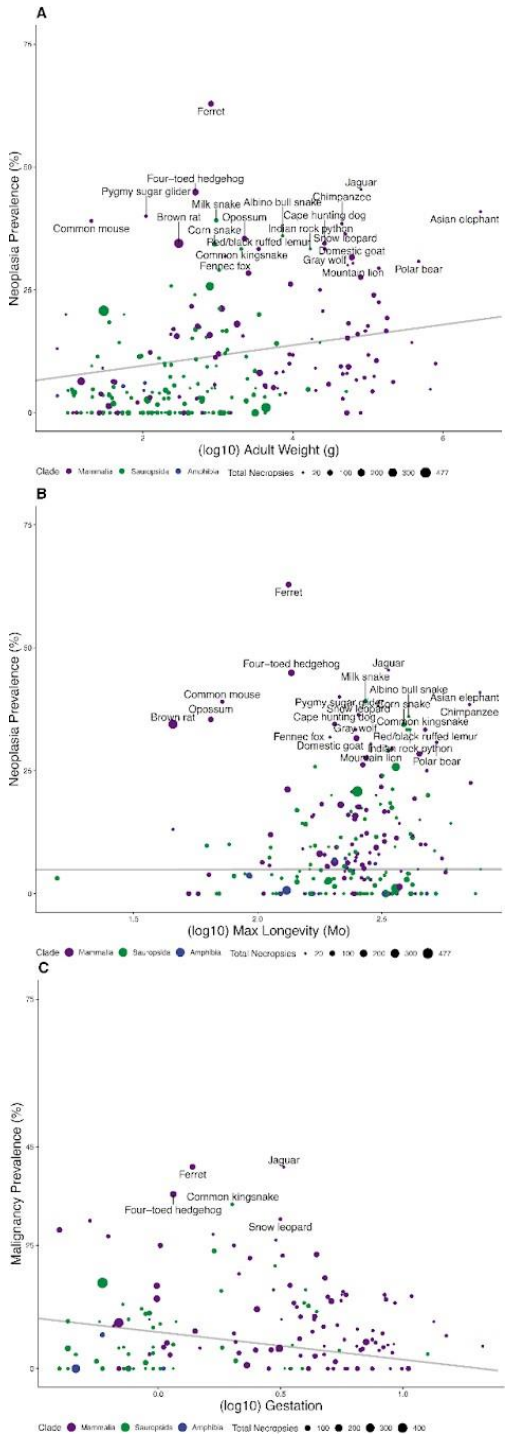

Figure 3. Significant life history factors associated with neoplasia and malignancy prevalence. **A.** Larger organisms have a higher neoplasia prevalence than smaller organisms (2.1% neoplasia per Log_{10}g adult body mass, $p = 0.007$, $R^2 = 0.18$, $\lambda = 0.46$). **B.** Longer lived organisms also have more neoplasia (0.01% neoplasia per Log_{10} month lifespan, $p = 0.02$, $R^2 = 0.16$, $\lambda = 0.34$). **C.** Organisms with longer gestation times have a lower malignancy prevalence (-5.65%

malignancies per Log_{10} months, $p = 0.02$, $R^2 = 0.01$, $\lambda = 0.41$). When controlling for adult body mass, organisms with longer gestation times also have fewer neoplasms at death (-5.30% neoplasia per Log_{10} months, $p = 0.1$).

In vitro DNA damage response

We tested primary fibroblasts from 15 species for their response to DNA damage (Fig. 4; Suppl. Figs. 45-60). Mammalian cells have two primary mechanisms to address DNA damage: cell cycle arrest to allow for DNA repair and apoptosis (Abegglen et al., 2015; Tian et al., 2015). In a previous study, we documented that low prevalence of death from cancer in elephants was correlated with enhanced DNA damage response (Abegglen et al., 2015). Therefore, we hypothesized that DNA damage response would predict malignancy. Figure 4 shows the predicted trend for both neoplasia and malignancy prevalence, though they are not statistically significant. We also analyzed responses to lower doses of ionizing radiation and increasing doses of a chemotherapeutic drug (doxorubicin). No association with response and neoplasia or malignancy was observed (S45 - S60)

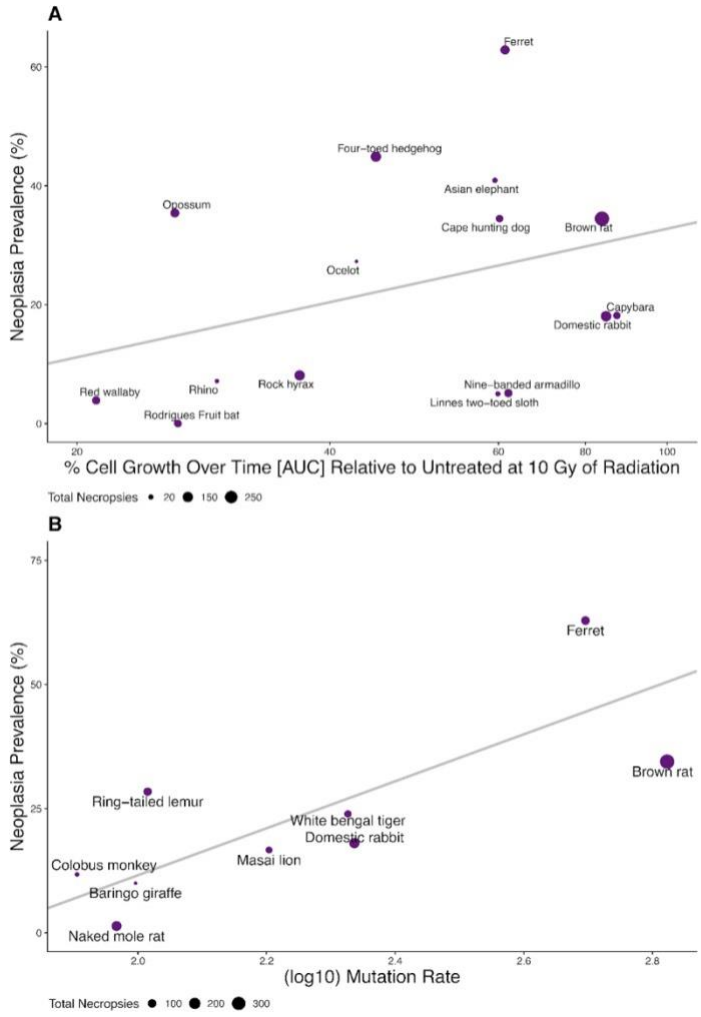

Figure 4. A. % Cell Growth Over Time [AUC] Relative to Untreated at 10 Gy of Radiation (plotted and analyzed on a Log_{10} scale) as a predictor of neoplasia prevalence in species' fibroblast cell lines. (30.89% neoplasia per Log_{10} Cell Count Area Change, $p = 0.22$, $R^2 = 0.011$, $\lambda = 6.6 \times 10^{-5}$) **B.** Log_{10} Mean Mutation Rate as a predictor of neoplasia prevalence (47.26% Single Base Substitution per Genome per Year, $p = 0.0059$, $R^2 = 0.96$, $\lambda = 1.00$).

Age as a Cancer Risk Factor in Animals

Age is the single biggest risk factor for the development of cancer in humans (White et al., 2014). Most mechanisms of somatic maintenance, including immune cell surveillance, DNA damage response, and telomere shortening, decrease in efficacy as we age (Campisi, 2005a, 2005b; Garinis et

al., 2008; Hasty et al., 2003; Kirkwood & Austad, 2000). To test if observed neoplasms in animals under human care may be due to the animals living beyond their natural lifespans, we plotted the age of the animals with neoplasia at death, compared to the animals that died without neoplasias, scaled by their average lifespan (Fig. 5). The vast majority of animal deaths with neoplasia diagnoses occur before the average lifespan in most animals. Only amphibians seem to be developing more neoplasias as they live past their normal lifespan under human care(Fig. 5C). The distribution of tumor diagnoses across lifespan in these three clades also demonstrates that cancer is not limited to a disease solely of extended lifespan, and in sauropsids, neoplasia is not particularly a disease of old age (Fig. 5B).

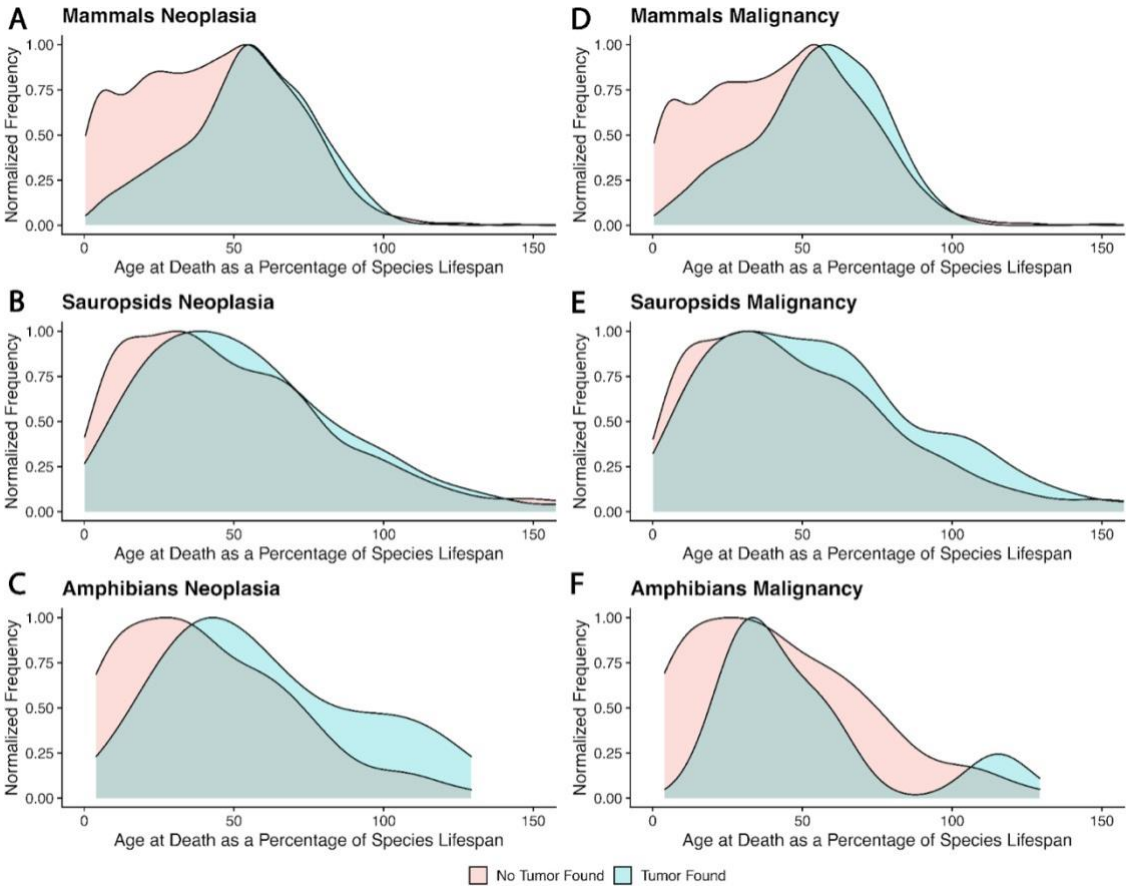

Figure 5. The density distribution and corresponding locally weighted regression (LOESS) of ages at death in animals with neoplasia versus non-neoplasia, adjusted for each species' lifespan as specified in PanTHERIA. While the distributions of ages at death are different between necropsies showing neoplasia versus those that don't (Two Sample Kolmogorov-Smirnov Test: Mammals: $D=0.11$, $p = 1.81 \times 10^{-6}$; Sauropsids: $D= 0.18$, $p = 4.48 \times 10^{-8}$; Amphibians: $D=0.5$, $p = 0.011$), we found few neoplasias that could be explained by an organism living an extraordinarily long time in captivity, except in amphibians.

Phenotypic Models of Cancer Risk

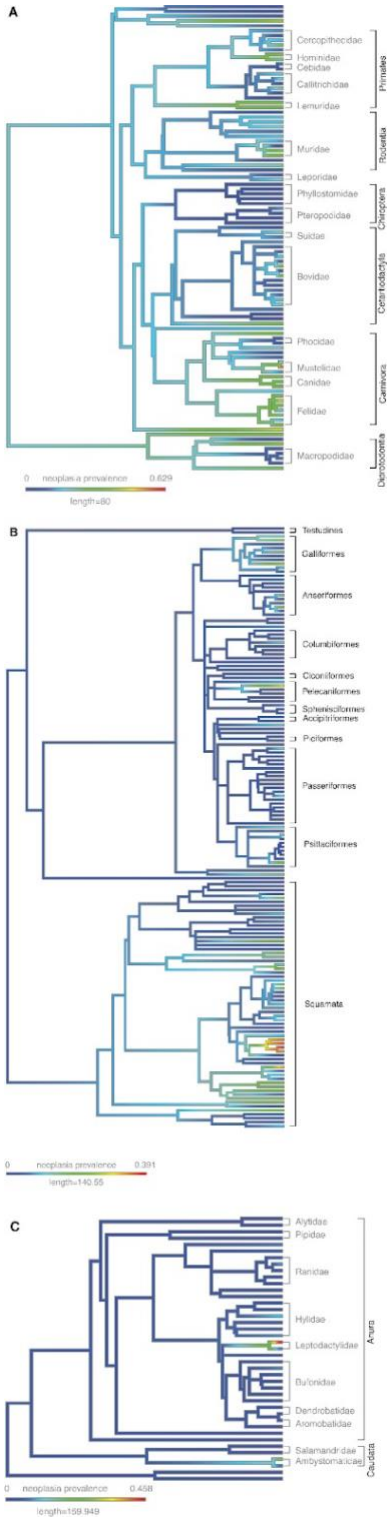

Figure 6. Cladogram depiction of cancer incidence within **A.** Mammals, **B.** Sauropsids, and **C.** Amphibians. Cladograms with the species labels at each tip

can be found in Suppl. Fig. 63. Heat map coloration indicates relative prevalence of cancer within each branch, illustrating the diversity of neoplastic disease amongst closely related species. The scale is the same for each panel so that the differences between the clades are apparent.

Evolution of cancer suppression and susceptibility

Comparative phylogenetics provides a wealth of computational tools to model species' trait evolution across a phylogeny (Felsenstein, 1985b). To explore how cancer susceptibility evolved across the tree of life (Fig. 6), we fit three of the most common phenotype evolution models (Ornstein-Uhlenbeck, Brownian Motion, and Early Burst) to neoplasia prevalence as a continuous trait. We found that a model of stabilizing selection on neoplasia prevalence (Ornstein-Uhlenbeck) fits the distribution of neoplasia prevalence the best (Supp. Tab. 7). Malignancy prevalence evolution is also best explained by the Ornstein-Uhlenbeck model of sudden shifts followed by stasis in the phenotype.

Methods

Analysis of Veterinary Necropsy Records

We collected necropsy records with permission from 99 zoological institutions, aquariums, and other facilities that house animals under managed care. Necropsies had all been conducted by board certified veterinary pathologists who specialize in nondomestic species, and identified if a neoplasia was discovered during a post-mortem examination. We used a terminology dictionary (Supplemental table 3) to distinguish benign from malignant neoplasms based on the diagnoses in the necropsy reports. We excluded neonatal records to reduce bias from high levels of neonate and infant mortality

that is common in many species. Because only common names were recorded for most records, we developed a tool, kestrel, to translate common names into scientific names which is available at https://pkg.go.dev/github.com/icwells/kestrel.

All of the institutions that provided prior approval for the use of their data in these analyses are Association of Zoos and Aquariums (AZA) accredited. AZA accreditation encourages the institution to perform a necropsy on all animals that die under their care to determine cause of death and to monitor morbidity and mortality of each species. Furthermore, each institution had IACUC approval with the Exotic Species Cancer Research Alliance (ESCRA) and the Arizona Cancer and Evolution Center (ACE) for the use of their deceased animal's records of animals with neoplasia for use in this study. Previous analyses included both necropsies for animals diagnosed with neoplasia and animals that were still alive (Vincze et al., 2022). In this study, we restricted our analyses to only necropsies - for both cancer and non-cancer diagnosed animals, because alive animals may harbor undetected cancer or might be eventually diagnosed with cancer, thus skewing estimates in cancer prevalence. The data for our analyses are available in supplemental file 1.

Comparative Phylogenetic Methods in Comparative Oncology

Interspecies comparisons must account for the shared ancestry and the constraint of natural selection on species' traits before a determinant of any correlations can be made. For the life history models of neoplasia and malignancy prevalence, the R programming (Development Core Team, 2011) packages "phytools" (Revell, 2012a), "ape" (Paradis et al., 2004), and "caper"

(Orme, Freckleton, Thomas, Petzoldt, Fritz, Isaac, & Others, 2013) were all used for phylogenetic comparisons and the handling of phylogenetic data. To accomplish this we wrote the function *pglsSEyPagel* which is built upon phytool's *pglsSEy* (phylogenetic generalized least squares for uncertainty in Y). pglsSEyPagel expands upon the pglsSEy function by adding the estimate of Pagel's lambda (Pagel, 1999) to the regression, rather than assuming it is fixed at 1 (i.e., Brownian motion). .

Testing for relationships with life history factors

We extracted data for maximum lifespan, adult body weight, basal metabolic rate, gestation length, litter size, time to sexual maturity, and growth rate from PanTHERIA (Jones et al., 2009). We used a weighted phylogenetic regression to control for non-independence of phenotypes (e.g. neoplasia prevalence) in closely related species. We report the phylogenetic signal, lambda, for each analysis, along with the p-value and R^2. A single phylogenetic tree encompassing the three clades was collected from timetree.org. We pruned the tree to the 327 species in our data set using the setdiff and keep.tip/drop.tip functions in the APE R package. Estimates for neoplasia and malignancy prevalence are more accurate in species with more necropsies. To address the differences in number of necropsies, and to limit the noise from prevalence estimates based on few individuals, we weighted the species data points by the square-root of the number of necropsies records we have. Our R code for all analyses and figures included in this manuscript is freely available at https://github.com/zacharycompton/cancerAcrossVertebrates.git . In addition, we

only analyzed species for which we had at least 20 necropsy results (previous studies had used 10 (Abegglen et al., 2015) or 20 (Boddy et al., 2020b; Vincze et al., 2022) for the lower bound number of individuals). The main *pglsSEyPagel* analyses were done with all species together, including mammals, sauropsids and amphibians. In the analyses of litter size and gestation time, we also tested for a relationship with neoplasia prevalence in mammals alone. We carried out a total of 28 *pglsSEyPagel* analyses. To control for multiple testing, we used a false discovery rate (FDR) of 10%.

DNA Damage Sensitivity Assays

Established, primary cells from mammals were obtained from San Diego Zoo Wildlife Alliance (Capybara, Linne's Two Toed Sloth, Red Necked Wallaby, Rock Hyrax, Rodrigues Fruit Bat, Six Banded Armadillo, Southern White Rhino, and Virginia Opossum) or generated at Huntsman Cancer institute from tissues collected from African Pygmy Hedgehog, Domestic Rabbit, Leopard, Asian Elephant, and Cape hunting dog, Brown rat (Cell Applications) and Normal Human Dermal Fibroblasts (Lonza) were commercially available. Lonza (Normal Human Adult Dermal Fibroblasts). Detailed information on culture conditions, primary donor demographics, and passage numbers can be found in the supplementary information. Cells were seeded in 96-well plates at 2,000 cells per well in cell growth media and allowed to adhere overnight. The following day, doxorubicin was added at one of four concentrations (0μM [DMSO vehicle control], 0.11μM, 0.33μM, and 1μM). Each condition was tested in triplicate in three separate experiments. Cell proliferation and apoptosis were measured by

real-time fluorescence microscopy (IncyCyte, Sartorius) at two-hour intervals for three days. Apoptosis was measured using a fluorescent cell death marker, Annexin V Dye (Sartorius). Images were processed and analyzed using IncuCyte software. The number of dead or dying cells were identified by counting Annexin V positive cells. In addition, cell count overtime was calculated using IncuCyte cell-by-cell software. To measure response to radiation-induced DNA damage, cells were irradiated with one of four doses: 0Gy, 0.4Gy, 2Gy, and 10 Gy. Radiation dose was delivered using an RS-2000 X-Ray Irradiator (Radsource). Cells were then seeded in 96-well plates in cell growth media containing Annexin V Dye (Sartorius). Cells were imaged by real-time fluorescence microscopy (IncuCyte, Sartorius) at two-hour intervals for five days. We observed little to no apoptosis of irradiated fibroblasts, as has been previously reported for this cell type (reference). We estimated cell cycle arrest by normalizing the cell count of irradiated cells to untreated cells by dividing the area under the curve (AUC) of cell count over-time for treated cells by the AUC of cell count over time for the untreated (UT) cells. We converted that number into a percentage that represents the percent of cell proliferation relative to untreated cells. We then tested if this normalized amount of cell growth was predictive of neoplasia prevalence using the phylogenetically controlled *pglsSEyPagel* regression (Fig. 4).

Discussion

We estimated cancer prevalence across a wide range of tetrapod species that includes mammals, amphibians, reptiles and birds. Importantly, and contrary

to previous studies, our analyses highlight limitations to Peto's paradox, by showing that large animals do tend to get somewhat more neoplasms, and malignancies when controlling for gestation time, compared with smaller animals. This is particularly apparent when we control for the fact that animals with longer gestation times tend to get both fewer neoplasias and fewer cancers. However, large animals only get slightly more cancer than small animals. Whether or not they get as much cancer as one would expect from their body size and longevity depends on the model one uses to predict cancer prevalence as a function of body size (Calabrese & Shibata, 2010; Caulin et al., 2015). We hypothesize that animals with a longer gestation time than would be expected for their body size, may be investing more resources to control cell proliferation, and thereby reducing their vulnerability to cancer, compared to animals with shorter than expected gestation times.

Cancer prevalence across species varies greatly. Here we have used the largest collection of species to date, and expanded our analyses beyond mammals (Abegglen et al., 2015; Boddy et al., 2020b; Vincze et al., 2022), to test for patterns in cancer prevalence. We only include species with at least 20 necropsies (median 35), compared with 10 individuals per species in our original study (Abegglen et al., 2015), and weighted species more in our regression analyses if their cancer prevalence estimate is more accurate because it is based on more necropsies. This revealed adult weight and gestation time as significant predictors of neoplasia and malignancy prevalence. The fact that neoplasia prevalence seems to evolve by sudden shifts followed by stabilizing selection

(the Ornstein-Uhlenbeck model of phenotypic evolution) is consistent with life history theory predictions that investment in somatic maintenance should be under selection in specific ecological conditions, rather than drifting neutrally consistent with random Brownian motion. Some of the variation in cancer prevalence is still noise, due to estimating cancer prevalence from tens of individuals. However, much of that variation comes from the vast diversity of species across amphibians, reptiles, birds and mammals. We have explained only a small portion (~20%) of the variation in species vulnerability and suppression of cancer. There is clearly more to be discovered.

Peto's Paradox is based on the expectation that large, long-lived animals should get more cancer because they have more cells that exist for a longer amount of time, increasing the likelihood that cancer will arise. Although adult body weight is positively correlated with both neoplasia and malignancy prevalence, partially resolving Peto's paradox, the effect size is much larger for neoplasia (3.8% neoplasia per $Log_{10}g$) than for malignancies (0.68% malignancies per $Log_{10}g$). There may be several explanations for this. The simplest being that malignancies are less common than neoplasias, which include both benign and malignant neoplasms. This reduces the statistical power and the expected size of the effects. However, the blunted relationship between body size and malignancy prevalence may also be due to natural selection acting to evolve mechanisms to suppress malignant transformation. Cancer suppression mechanisms are likely to have been under stronger selection among these larger and longer-lived organisms because it was more critical to suppress

cancer for longer in order for these organisms to successfully reproduce. Thus, we might expect a relatively constant cancer rate across species with more cancer suppression mechanisms in large, long-lived organisms (Abegglen et al., 2015; A. Aktipis, 2020; Glaberman et al., 2021; Sulak et al., 2016; Tollis et al., 2019; Vazquez et al., 2018; Vazquez & Lynch, 2021), and fewer in small, short-lived organisms.

Further, there are at least four transitions in neoplastic progression that natural selection might alter to increase the survivability of cancer in a species: 1) initiation of a neoplasm, 2) transformation of that neoplasm into malignancy (*i.e.*, invasion through the basement membrane), 3) metastasis, and 4) death caused by the cancer. Our data bear on the first two transitions. Specifically, we quantify the prevalence of neoplasms in a species, the prevalence of malignant neoplasms, and the proportion of neoplasms detected that are malignant. However, the selective pressure of cancer is ultimately due to its effects on mortality, and so quantifying the prevalence of cancer as a cause of death would be more relevant for evolutionary studies of comparative oncology (Vincze et al.,2022). The inclusion of cross-species functional assays highlighted in Fig. 4 demonstrates above all that there is tremendous variation in the cellular responses to radiation induced DNA damage. This is the first time functional assays to measure DNA damage response have been paired with species' cancer prevalence. While response to DNA damage was not a significant predictor of neoplasia or malignancy prevalence at 4 or 10 grays, the trend follows our hypothesis that sensitivity to DNA damage may be one mechanism of

cancer suppression (Abegglen et al., 2015). The variation observed in our DNA damage response assays suggests that many species may use other mechanisms of cancer suppression (such as immune surveillance or other forms of DNA damage and somatic mutation), thereby obscuring a simple relationship between our measurements and neoplasia or malignancy prevalence.

Insights from comparative oncology for human cancers

Species with a high prevalence of particular cancers may help to generate targeted studies to elucidate the biological basis of those cancers, help draw informative parallels to particular cancer syndromes in humans, and serve as more realistic models for studying those cancers. For instance 46% of the malignant tumors diagnosed in the opossum were lung adenocarcinomas (Suppl. Tab. 8), which is a leading cause of human cancer mortality in the United States (Islami et al., 2021). Hedgehogs may hold insights for colorectal cancer, the third leading cause of cancer mortality in the US, and ferrets may help us understand lymphoma. These spontaneous cancers may be more similar to human cancers than genetically manipulated mice, though that remains to be tested. There is an exciting opportunity to discover the mechanisms for suppressing cancers in species with few to no observed neoplasms, or those that seem to prevent neoplasms from progressing to malignancy (Fig. 1). For example, the paucity of neoplasms in dolphins and porpoises may be due to a legacy of once having had large, long-lived cetacean ancestors that were under strong selection to suppress cancer (Tollis et al., 2019). Our earlier analysis of cancer gene evolution in cetaceans found evidence of positive selection in a large number of tumor

suppressor genes and proto-oncogenes (Tollis et al., 2019).

We previously found that animals that live longer than would be expected for their body size, like bats, tend to have more copies of tumor suppressor genes (Tollis et al., 2020). In support of these observations, we find 9 bats, with an average lifespan of 16 years, have low neoplasia prevalence. We had hoped to discover species that are able to prevent malignant transformation by finding species that get a fair number of benign neoplasms, but few to no malignant neoplasms. The common paradigm in understanding the evolution of cancer suppression emphasizes the importance of protecting against tumor initiation. However, mechanisms that suppress malignant transformation could be similarly important in maintaining an organism's fitness. Unfortunately, only a few species in our dataset fit that description. The species with the lowest proportion of malignant to benign neoplasms was the common squirrel, with only 12% of their tumors being malignant.

Challenges for Comparative Oncology

There are a number of potential sources of bias in comparative oncology data. The protection against predation that zoological institutions offer fast life history animals may be extending their lifespan, and thereby unmasking a vulnerability to cancer at an age that they would never attain in the wild. However, Fig. 5 shows that the neoplasms were diagnosed prior to average lifespan in most cases, suggesting the extended lifespan due to managed care is not a large factor in these data. In fact, one surprise was that neoplasms appear in birds and reptiles at relatively young ages, although birds are known to be

prone to virally induced cancers (Beard, 1963).

Our data results from the combination of the intrinsic cancer susceptibility of a species with the effect of the artificial conditions of managed populations, which is sometimes called an evolutionary mismatch (Schulte-Hostedde & Mastromonaco, 2015). These animals were generally protected from predators, provided with veterinary care, had different diets and exercise from their wild counterparts, many lived in an urban environment, and interacted with different species and microbes than a free-ranging animal. It is striking to us that four of the species with the lowest prevalence of neoplasias in our dataset, the gray squirrel, the common dormouse, the striped grass mouse, and the common field vole are all from wild, urban populations. These necropsies come from the London Zoo which has a policy of performing a necropsy on any animal they recover that dies on its grounds, not just the animals under its care. This is a hint that cancer may well be less common in the wild.

If the “gross” cause of death was obvious for an animal, an institution may not have submitted the animal’s samples for histopathology, and would not be included in our data collection. Similarly, if a particular type of neoplasia is difficult to detect in a necropsy (including some leukemias and intracerebral tumors), or was only present at a microscopic level, it may have been undercounted. The functional assays were limited to fibroblast cell lines for the species for which we could obtain samples. Limited sample availability precluded the ability to control for biological factors such as age, which we expect to influence DNA damage sensitivity.

The Future of Comparative Oncology

In the future, it will be important to collect additional data to validate our discoveries of species with particularly low and high cancer prevalence, such as those highlighted in Fig. 1. Several life history traits, such as basal metabolic rate, may explain cancer risk but with BMR measured in only a few species, we lacked statistical power to detect a relationship with neoplasia or malignancy prevalence. Here we have dramatically expanded the amount of data on cancer prevalence in non-human animals, but this must continue to be built upon if we are to match the robustness seen in human cancer epidemiology. In particular, much could be learned from analyzing the age-incidence curves of cancer (Vincze et al., 2022; Watson, 1977), but that would require significantly more individuals for each species.

One of the most important holes in comparative oncology is cancer data on wild animals. Gathering data from free-ranging populations is challenging, as it is difficult to detect cancer due to the animals decaying or being eaten before they can be necropsied. Additionally, accurate age estimates are much more difficult to obtain in wild populations compared to those managed by humans. However, wild animal populations would greatly enhance the field of comparative oncology by validating species that have low cancer prevalence and testing for evolutionary mismatches for animals in captivity.

Conclusion

Cancer is a problem of multicellularity (Aktipis C. Athena et al., 2015). While we found a relationship between both body mass and gestation time with cancer prevalence, we are just beginning to discover patterns of cancer susceptibility and cancer defenses across species. It is likely that evolution has developed a variety of mechanisms for preventing cancer. The discovery of particular species with extremely low neoplasia prevalence provides opportunities for elucidating cancer suppression mechanisms that are compatible with life and reproductive success. Investigation of species with extreme vulnerability to a particular cancer may also help us understand those cancers as well as human syndromes that predispose to those cancers. We hope to learn from nature how to better prevent cancer in both humans and non-human animals.

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Author Contributions

AMB, HD, TMH, DM, MG and VH collected the data. SR, ZC, JL, DM and VH curated the data. ZC, SR, WM, AP, BM, EM, SA, and CB carried out the analyses. MG, BT, SS provided veterinary pathology support and TMH provided veterinary supervision. BT created and provided the data dictionary for distinguishing benign from malignant disease. RK, KN and MW generated

fibroblast cell lines and functional data generated with them, supervised by LA and JS. ZC, CCM and AA wrote the manuscript. CCM, AMB, AA, MT, TAG, LA, JS supervised the research. All authors edited the manuscript.

CHAPTER 4

NEOPLASMS IN PRIMATES: AN EVOLUTIONARY PERSPECTIVE ON CANCER PREVALENCE IN NON-HUMAN PRIMATES

In Defense of Close Cousins

You may be surprised how tense a subject primate evolution still is. Well, at least a specific region of that primate phylogeny. A particularly nauseating framework has been discretely nurtured when it comes to the introduction of evolutionary theory to students. I was perfectly content with the original concession that evolutionists granted to theology, allowing us to explain the deep history connecting the origin of life and humans, leaving the very origin of life largely in their domain. But it seems we have given the mouse a cookie in this respect. At the same time that many universities are providing entire degree programs in human evolution many have quite graciously offered a new concession. Two types of evolution; evolution that describes humankind's divergence from our primate ancestors and then all the apparently more palatable evolution. That being the case I felt this a special preface for my comparative oncology piece on primate cancers. I agree, there is a reason we should be particularly interested in comparative anatomy, physiology, and disease in these species. I just wish to ensure we mutually agree on why that is. The awkwardness in primate evolution is born from a hesitancy Charles Darwin himself nurtured in *On the Origins of Species* with his intentionally opaque “light will be thrown on the origin of man and his history”(Darwin, 1872). We don’t know for sure which of Charles Darwin’s transcontinental observations were the

most crucial to his revelations of natural selection and common ancestry. History seems to have made that decision for him. The phenotypic variations in the beaks of the finch populations scattered across the Galapagos archipelago are universally affixed to undergraduate lectures on evolution. It is even likely that there was no single observation that solidified the view of life revealed to him. Given the language and structure of *On the Origins* it certainly seems the portrait was only completely understandable when viewed as a whole. Whatever may have sparked his personal revelation on the veracity of evolution, we all have the privileged opportunity to have the same when we gaze on the faces of our closest phylogenetic cousins.

Studies of physiology and behavior in primates have solidified a great deal of understanding of our own mechanics. Despite the incredible degree of homology between ourselves and primates, even those more distantly related than the chimpanzee, infrequent attention has been given to the type and prevalence of their cancerous tumors. Primates have long held high value in scientific studies for the use of drug development and efficacy. This value is derived from our close evolutionary relationship with them but rarely has the value in understanding primates' burden of disease and our evolutionary relationship with them been so explicitly described as it is in what follows.

Abstract

Studying cancer from an evolutionary perspective can lead to important theoretical and applied insights, however little is known about the prevalence of cancer among non-human primates. Primates are the closest living relatives to

humans, however the Primate lineage is phenotypically diverse, with wide variations in evolutionary and life history characteristics. By leveraging comparative phenotypic data with incidence records of neoplastic disease, we have constructed a dataset of 2,095 individuals across 35 species, and explore cross-species cancer risk within Primates. We suggest a life history theory framework to help elucidate the variations in observed cancer prevalence across non-human primates, wherein long-lived, large-bodied animals invest more energy in somatic maintenance (i.e. cancer defenses) to maintain their cellular body. Additionally, functional studies performed *in vitro* using isolated and cultured primary fibroblast cell lines from representative species show that resistance to cellular death is correlated with certain life history characteristics. Combining large-scale cancer incidence records and functional assays can provide useful insights into the ecological and cellular dynamics of cancer in our closest living relatives and ourselves.

Introduction

Cancer is a problem for all multicellular organisms, not just humans (Aktipis C. Athena et al., 2015). Little is known about cancer in non-human organisms, including our nearest relatives - primates. Without large-scale data on neoplasia prevalence and malignancies in primates, it is difficult to determine what aspects of cancer are peculiar to human biology and lifestyle factors, and what aspects are a legacy of our shared ancestry with other primates.

The evolution of complex multicellularity requires a delicate balance between cellular-level fitness and organismal fitness, and cancer can be viewed

through the lens of a breakdown of this cooperative agreement(Aktipis C. Athena et al., 2015). The forces of evolution act upon multiple levels of selection, and as organisms gain more and more complexity, cancer suppression mechanisms must concurrently evolve to maintain organismal fitness(Nedelcu & Caulin, 2016). Sir Richard Peto postulated in 1977 that the risk of cancer should scale with body size (i.e., larger number of cells within an organism) and length of lifespan (i.e., number of cellular divisions that increase the chances of acquiring a neoplastic mutation), however there must be an evolutionary pressure to evolve mechanisms to prevent neoplastic growth, a phenomenon known as 'Peto's paradox'(Peto, 2016). Recent comparative studies have verified this observation and have found that typically, large-bodied and longer-lived organisms experience less cancer than their smaller counterparts(Tollis, Schiffman, et al., 2017; Vincze et al., 2022).

Primate species vary widely in a number of phenotypic characteristics thought to impact lifetime cancer prevalence including body size, lifespan, and growth rate. Previous work has suggested that cancer risk should increase with the number of cells in a body and the number of years that they must be maintained. This seems to be true within species, and well documented in humans(Nunney, 2018), but initial observations across mammals suggest that there is no relationship between cancer prevalence and either body size or lifespan(Abegglen et al., 2015). Others have emphasized the relevance of evolutionary tradeoffs between reproduction and cancer suppression(Boddy et al., 2015).

Non-human primates have long been invaluable in comparative biology to

study behavior, senescence and even infectious disease(Chang et al., 2009; Flynn et al., 2015; Herbig et al., 2006; Sapolsky, 2005, 2006; Wolfe et al., 1998). Based on the diversity of life-history characteristics and the phylogenetic similarity with humans, the Primate lineage is an ideal system to explore incidences of cancer and the relationships between life-history characteristics that may have influenced the evolution of tumor suppression mechanisms. The majority of previously conducted studies have been constrained by investigations into specific tumor types, limited by particular species or locations, and typically are constituted by a low sample number of individuals. All of these introduce significant limitations to our ability to identify patterns and generalize observations.

In this work, we present data from over 2,095 individuals belonging to 9 unique primate families, across 25 years of collected histopathology and necropsy reports from multiple zoological institutions, wildlife sanctuaries and veterinary facilities. This represents the largest dataset on primate cancers that has been collected to date.

Methods

Neoplastic prevalence was established by using previously collected pathology records from multiple zoological, veterinary, and animal sanctuary databases. 23 years of necropsy data collected by the Northwest Zoopath, Duke Lemur Center, Drury, Michigan State University was compiled and tumor prevalence was calculated for 35 primate species. Pathology records compiled from these institutions represent veterinarian medical records from both routine

health care and postmortem necropsy surveys, and were conducted by professional veterinary pathologists. Pathology records were then paired with known life history variables, collected in the databases panTHERIA (Jones et al., 2009). All data for our analysis is provided within the supplementary information, as well as the aggregated life-history variables that were explored.

Statistical Analysis

Neoplasia and malignancy prevalence were calculated and compared with multiple life history traits, including adult body mass, lifespan, basal metabolic rate, growth rate and gestation length. For all plots, the R packages caper, ape, and phytools were used to construct Phylogenetic Least Squares (PGLS) models to account for phylogenetic interdependence(Orme, Freckleton, Thomas, Petzoldt, Fritz, Isaac, & Others, 2013; Paradis et al., 2004; Revell, 2012a). All of the reported statistical values are representative of a linear fit post-PGLS analysis. Fisher's exact tests and Wilcoxon signed rank tests were both used for the determination of a sex bias in neoplasia and malignancy prevalence.

Functional Studies

Primate cells were obtained as established cell lines from San Diego Zoo (chimpanzee, gorilla, orangutan, ring-tailed lemur, baboon, and spider monkey), Duke Primate Institute (mouse lemur), Kansas City Zoo (Cotton-top tamarin) and Lonza (normal human adult dermal fibroblasts, NHDF). Detailed information on culture conditions, primary donor demographics, and passage numbers can be found within the supplementary information.

Cells were seeded in 96-well dishes at a concentration of 1,000 cells per

well in 100ul cell-specific media and allowed to adhere overnight. The following day, doxorubicin was added in four treatment concentrations: 1uM, 0.33uM, 0.11uM and 0uM. All treatment conditions were tested in triplicate. Response to DNA damage was measured using two distinct fluorescent cell death markers. The first, Annexin V Green Reagent, binds to phosphatidylserine phospholipids that become exposed early in the cell death process as the cell membrane loses stability. The second, Caspase 3/7 Green Reagent, is triggered by activated caspases and is indicative of caspase mediated apoptosis. Cells were imaged using an IncuCyte at two hour intervals for four days. IncuCyte images were analyzed to determine the number of living and dead cells over time.

Results

Figure 1: The variation in neoplasia and malignancy prevalence across 35 primate species. Green bars represent neoplasia prevalence and purple bars represent malignancy prevalence. The two species highlighted with green squares, the Dusky leaf monkey and the Blacked-capped squirrel monkey, have zero reported neoplasms.

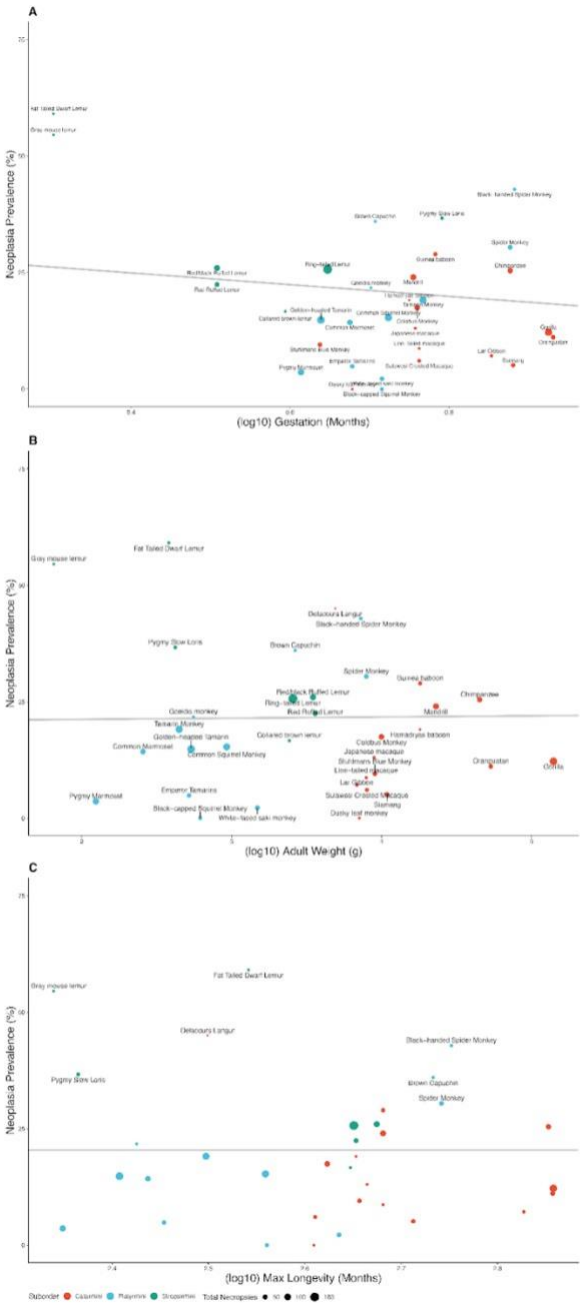


Figure 2: A) Log_{10} Gestation length (months) as predictor of neoplasia prevalence (p=0.47, λ =0.99), B) Log_{10} Adult weight (grams) as predictor of neoplasia prevalence (p=*0.93* , λ = 0.99), C) Log_{10} Maximum longevity (months) as a predictor of neoplasia prevalence (p= *0.86* , λ =0.99).

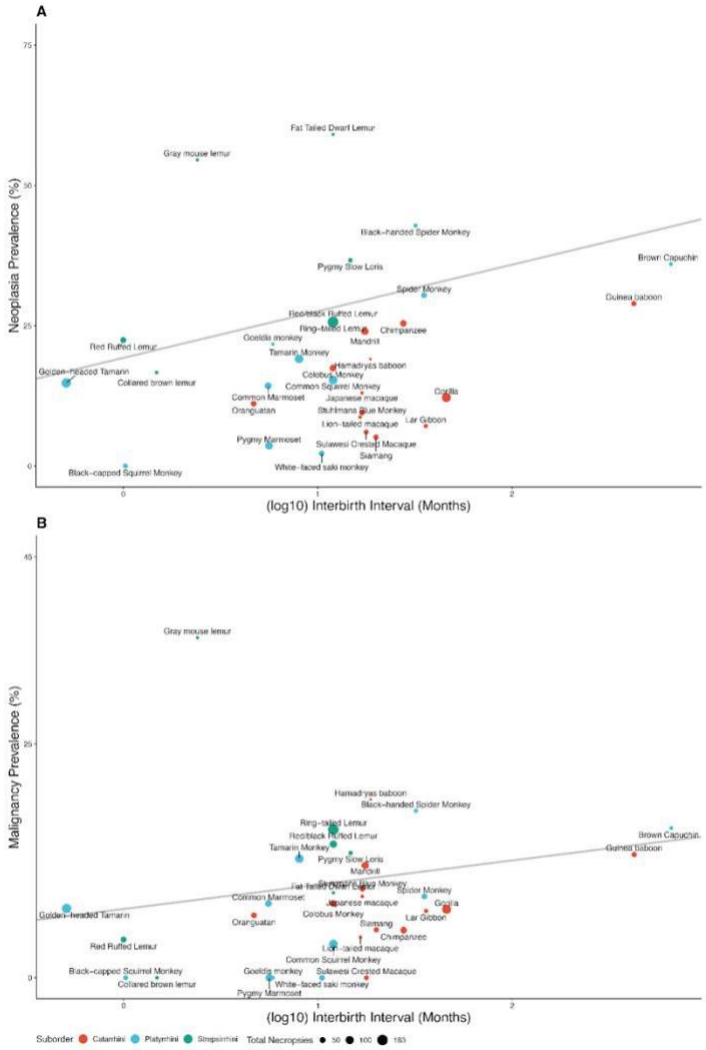


Figure 3: Log_{10} Interbirth interval (months) as a predictor of A) neoplasia prevalence (p=0.13, λ =0) and B) malignancy prevalence (p=0.01, λ =0).

Cancer rates across all species of non-human primate within our dataset show that the phenomenon of Peto's paradox remains within the primate lineage. Larger, longer-lived primate species, such as great apes do not suffer the same burden of neoplastic disease as smaller, shorter-lived primates. The lambda values in our phylogenetic least squares analyses indicate the significant strength of the phylogenetic signal in these life history models. The penetrance of

phylogenetic signal is indicative of the phylogenetic determinants of these life history traits and these species' cancer prevalence (Figure 2). Within primates, despite the two species where zero neoplasms were observed, there is less variation in cancer risk than when all mammals are compared(Boddy et al., 2020b; Vincze et al., 2022). Which is to be expected given the relatively young most recent common ancestor (MRCA) of all living primates existing only 66-69 million years ago.

The significance of interbirth interval as a predictor of malignancy prevalence, and a similar yet insignificant pattern shown for neoplasia prevalence suggest that more robust measurements of parental investment may point to clues on trade offs with somatic maintenance (Figure 3). Parental investment is a commonly studied trait in primates but is so far too conserved to specific primate species as to be applicable to the cancer data presented here(Bercovitch, 2002; Maestripieri, 2002).

Sex Bias in Primate Cancer Risk

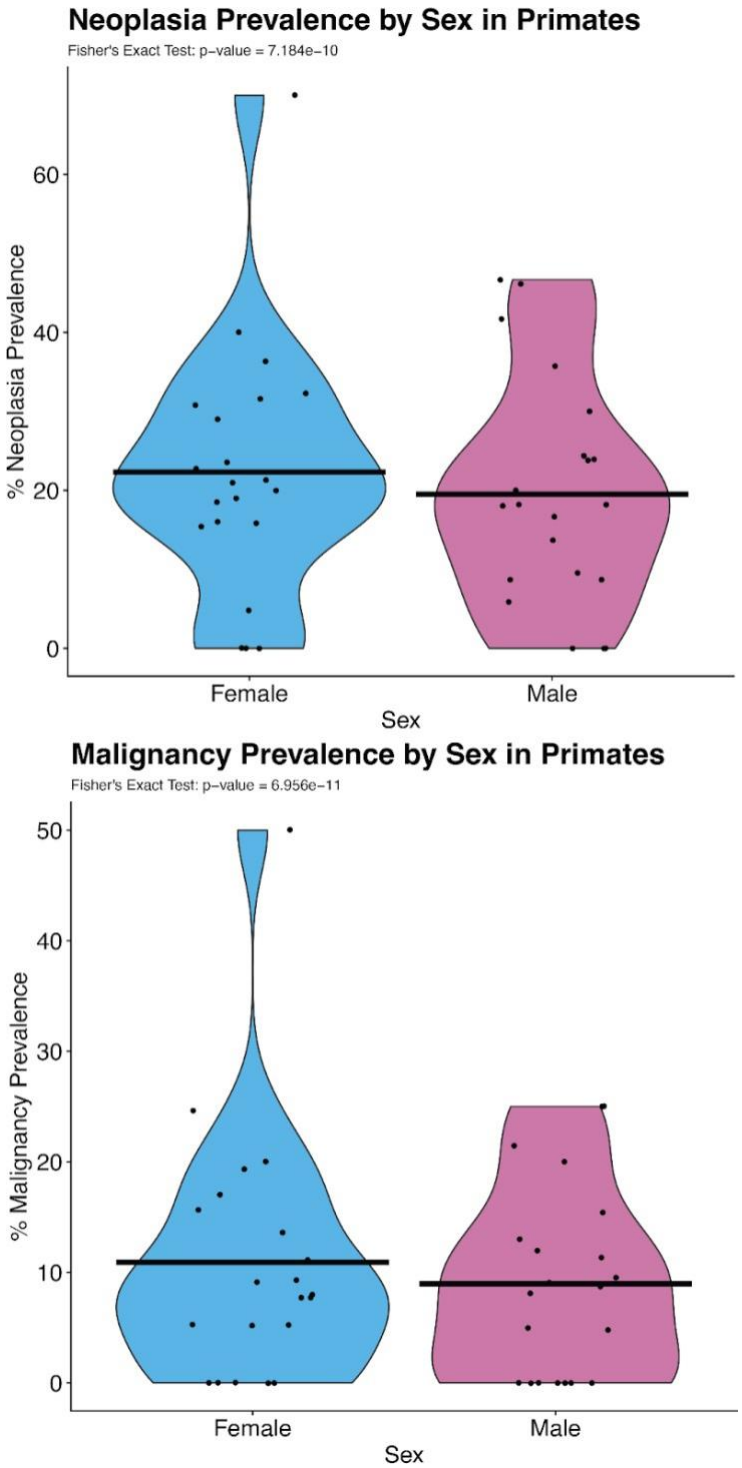


Figure 4: Violin plots of the sex specific prevalence of neoplasms and malignancies.

Discussion

In this paper, we report the largest known database of confirmed primate neoplastic prevalence, and explore potential trade-offs to cancer susceptibility and different life-history strategies. The database contains age and sex segmented data, as well as classifications of tumor type, location and malignancy. We have found that tumor prevalence has no relationship to body mass and lifespan, confirming Peto's paradox exists within the primate lineage. We examine multiple life history variables and their relationship to neoplastic prevalence. We also examine organismal age and how it relates to neoplastic prevalence, using both average wild lifespan and captive lifespan data. Our results show that neoplastic prevalence is not necessarily an artifact of extended lifespan as a result of captivity, but rather is most strongly correlated with phylogeny.

The significant female bias in both neoplasia and malignancy prevalence as shown in Figure 4 is the opposite of the pattern we observe in humans, with men burdening the higher risk. There are several intriguing differences between us and the rest of the primate taxon that may explain this. Differences in energetic investment in offspring offered by males and females provides a striking evolutionary hypothesis, especially given the discrepancy in investment that occurs pre-birth. A more proximate hypothesis could be the mismatch in reproductive cycles that many mammals face under human management.

Primates are our closest living relatives, with the chimpanzee genome sharing over 96.5% sequence similarity to humans. Despite these similarities, the

entire primate lineage has a wide variation within their evolutionary history and life history characteristics. This wide phenotypic variation paired with the phylogenetic similarity of our closest living relatives provides a unique opportunity to study human diseases from a comparative perspective. The hominin lineage split from the other primates approximately 6-7 million years ago, with humans diverging from the rest of the hominin lineage roughly 200-300 thousand years ago (Schwartz & Tattersall, 2010; Wood & Richmond, 2000). Humans and their hominin relatives share an evolutionary history, and in fact experience disease in a similar way. Among these similarities are their response to mutagens and resulting DNA damage. We show that adult body mass and lifespan are not correlated with cancer prevalence within the primate lineage, showing that despite the difference in total number of cells and cellular divisions, primates with larger bodies do not experience more cancer than smaller bodied primates. We suggest a life history theory framework to understand the differences in cancer incidence, with slow life history (*K*-selected) species having more capacity for responding to DNA damage and thereby cancer suppression mechanisms versus fast life history (*r*- selected) species.

Conclusion

By harnessing the information collected longitudinally from zoological institutions, primate sanctuaries, and research institutes, we are able to understand the breadth of differences in cancer incidence across species. Through examining these datasets and applying an evolutionary life history-based framework we are able to better understand the dynamics of cancer and

gain important insights into human biology. Further exploration into the field of comparative oncology is a worthwhile endeavor, and the myriad of cancer suppression mechanisms may be as diverse as the natural world that surrounds us.

CHAPTER 5

LIFE HISTORY AND CANCER IN BIRDS: CLUTCH SIZE PREDICTS CANCER

The Ghost of Giants

I cannot preface this manuscript any better than can be found in Arvid Agren's preface to his own book *The Gene's Eye View of Evolution* where he states "One of my biggest embarrassments in life is that I am such a poor naturalist. My botanical skills are distinctly average and my ornithological knowledge is downright appalling." (Arvid Ågren, 2021). The only addition to his bravery I can offer is that I cannot honestly attest to any personal interest in ever expanding my ornithological knowledge. I am too often within earshot of some graduate student who seems to know every variation of campus bird anyway. But like so many other topics in biology, my interest can be piqued when I am reminded of the all too envious, and all too epic, evolutionary history they boast.

My thinking on the evolution of cancer suppression mechanisms and the general regulators of somatic maintenance stems from viewing much of evolution as addressing a scaling problem. This framework predates me and is the basis of Peto's Paradox and the primary interest within comparative oncology to focus on species that underwent processes of gigantism sometime in their evolutionary history. Even with these species we consider our modern giants, many of them have a considerably smaller mass than their recent ancestors. However, while studies of gigantism have dominated much of comparative oncology we see that a study in miniaturization leads to many fascinating results.

Although birds have a great evolutionary distance from their theropod

dinosaur ancestors, they still provided a fantastic inverse to the classic comparative oncology question. While we have focused on how timely and how effectively natural selection may shape robustness in somatic maintenance while species increase in size, we now have the opportunity see how much time is required for natural selection to *realize* those mechanisms, while maybe necessary in distant ancestors, are no longer needed. Yagmur Erten and Hannah Kokko directly addressed this question with their 2021 preprint in which their model demonstrated that sufficient lags in evolution may explain why small bodied birds retain such an intense ability to suppress cancer.

The results highlighted in the following paper, especially now when laid in the context of *Cancer Across Vertebrates,* demonstrates that birds are one of the few taxonomic classes of animals that seem to preserve Peto's Paradox within both neoplasms and malignancies. The genomic analyses that are sure to come will provide exciting insights not only into the genes that govern the cancer suppression mechanisms in these species but how these genes compare to what we know about the vitality of known cancer suppression genes in mammals.

Abstract

Cancer is a disease that affects nearly all multicellular life, including birds. However, little is known about what factors explain the variance in cancer prevalence among species. Litter size is positively correlated with cancer prevalence in managed species of mammals, and larger body size, but not incubation or nestling period, is linked to tumor prevalence in wild birds. Also, birds that produce more elaborate sexual traits are expected to have fewer

resources for cancer defenses and thus higher cancer prevalence. In this study, we examine whether cancer prevalence is associated with a wide variety of life history traits (clutch size, incubation length, body mass, lifespan, and the extent of sexual dimorphism) across 108 species of managed birds in 25 different zoological facilities, sanctuaries, and veterinary clinics. We found that clutch size was positively correlated with cancer and neoplasia (both benign and malignant) prevalence, even after controlling for body mass. Cancer prevalence was not associated with incubation length, body mass, lifespan, or sexual dimorphism. The positive correlations of clutch size with cancer prevalence and neoplasia prevalence suggest that there may be life-history trade-offs between reproductive investment and somatic maintenance (in the form of cancer prevention mechanisms) in managed birds.

Introduction

Nearly all multicellular organisms are susceptible to neoplastic disease(Aktipis C. Athena et al., 2015; Effron et al., 1977a). Neoplasia is a disease consisting of uncontrolled cell division and growth, resulting ultimately in the formation of a tumor, as well as invasion or metastasis in case of malignant neoplasia (aka cancer)(C. A. Aktipis & Nesse, 2013; López-Lázaro, 2016). Over the past few decades, cancer research has focused on identifying different molecular pathways, hallmarks, and control mechanisms of cancer – all with the ultimate aim of improving cancer treatment(Bernards et al., 2020; Varmus, 2006).Evolutionary biology has also been an important component of cancer research over the last 50 years(C. A. Aktipis & Nesse, 2013; Dujon et al., 2020).

The ecological conditions under which organisms evolved have shaped their responses to various diseases, including cancer(Grunspan et al., 2018; Kapsetaki et al., 2022). Understanding why organisms differ in their ability to suppress cancer, as well as how they respond to neoplastic expansion, is a central question in comparative cancer research.

In general, life history trade-offs govern how organisms allocate time and resources to fitness components such as growth, self (or somatic)-maintenance, and reproduction(Charnov, 2003; Ghalambor & Martin, 2001). Somatic maintenance can include tumor suppression mechanisms such as cell cycle control and DNA damage repair. These trade-offs may help explain the variation in cancer prevalence across species. For example, long-lived species that invest in somatic maintenance over reproduction likely evolved enhanced mechanisms to suppress or evade cancer during their relatively long lifespans compared to short-lived species that invest heavily in reproductive effort rather than somatic maintenance(Harris et al., 2017). Peto's paradox predicts that bigger-longer lived animals would not be more vulnerable to cancer(Caulin & Maley, 2011; Roche et al., 2013; Tollis, Boddy, et al., 2017). Utilizing this life history tradeoff approach can both give us insight into the basic biology and origins of cancer and also provide opportunities to discover either universal or novel mechanisms of cancer suppression that could have clinical applications to humans.

Birds (class Aves) represent a diverse vertebrate clade with considerable variation in life-history characteristics. This makes birds a suitable system for investigating the correlation between cancer risk and certain phenotypic traits,

such as body mass and lifespan. Double-barred finches weigh on average just 9.5 grams, whereas greater rheas weigh on average 23 kilograms. Gouldian finches live on average up to six months, whereas salmon-crested cockatoos live on average up to 65 years (supplementary data). Birds also have a ZW genetic sex determination system, with females as the heterogametic sex, and therefore can also shed light on possible sex biases in health outcomes. For instance, female birds may be more susceptible to deleterious mutations promoting cancer development, whereas male birds may be protected by non-mutant versions of those alleles on their extra Z chromosome. This is known in humans as the two-X chromosome theory of cancer protection(Dorak & Karpuzoglu, 2012). If this two-chromosome theory is correct, we would expect female birds to have higher cancer prevalence than male birds.

Cancer prevalence in birds has been an area of ongoing study. Previous work reports that birds are amongst the vertebrates with the lowest cancer prevalence(Effron et al., 1977a; Madsen et al., 2017b; Pesavento et al., 2018). Within birds, there is much variation in cancer prevalence which may be explained by some phenotypic traits. For instance, Møller et al. surveyed free-living Eurasian birds post-mortem and found that, when analyzing at least 20 individuals per species, larger body size was correlated with tumor prevalence(Møller et al., 2017), while neither incubation nor nestling time were correlated with tumor prevalence(Møller et al., 2017). Separate studies have reported neoplasms (benign and malignant tumors combined) in bird species, either free-living or in human care(Effron et al., 1977a; Langohr et al., 2012;

Malka et al., 2005; Reece, 1992; Snyder & Ratcliffe, 1966; Speer, 2015; Stewart, 1966), but the prevalence of malignancy itself has not been measured before across bird species.

Clutch size could also be an important factor influencing the amount of energy devoted to somatic maintenance, including immune function, given the energetic trade-off between maintenance of a particular species' own body versus its offspring(Daan et al., 1996; Hanssen et al., 2005). There may also be a trade-off between reproductive investments and somatic maintenance(Boddy et al., 2015) such that sexually dimorphic or dichromatic species experience increased cancer prevalence(Fernandez & Morris, 2008) due to the somatic maintenance costs incurred by growing and maintaining these exaggerated morphological traits(Cherel et al., 1994; Klaassen, 1995; Moreno et al., 2001; Vézina et al., 2009). However, there has not been a study investigating the relationship between reproductive or sexually selected traits and cancer prevalence in birds.

To investigate the relationship between life history and cancer risk in birds, we combined trait-rich life-history databases with cancer prevalence data from veterinary records of 108 bird species under managed care. We hypothesized that the incredible diversity of life-history strategies observed across the class Aves can explain taxonomic differences in cancer risk in birds, due to the evolutionary trade-offs between growth, reproduction, and somatic maintenance. We test Peto's paradox (under the expectation that body mass does not explain variation in cancer prevalence) in birds, and investigate whether malignancy

prevalence or neoplasia prevalence is correlated with other avian traits such as incubation length, clutch size, and degree of sexual dimorphism and dichromatism. We also test for sex differences in cancer prevalence in birds, e.g. whether female birds (ZW sex chromosomes) have higher cancer prevalence than male birds (ZZ sex chromosomes). This study is the first to examine a wide range of life history traits in birds in order to predict cancer prevalence.

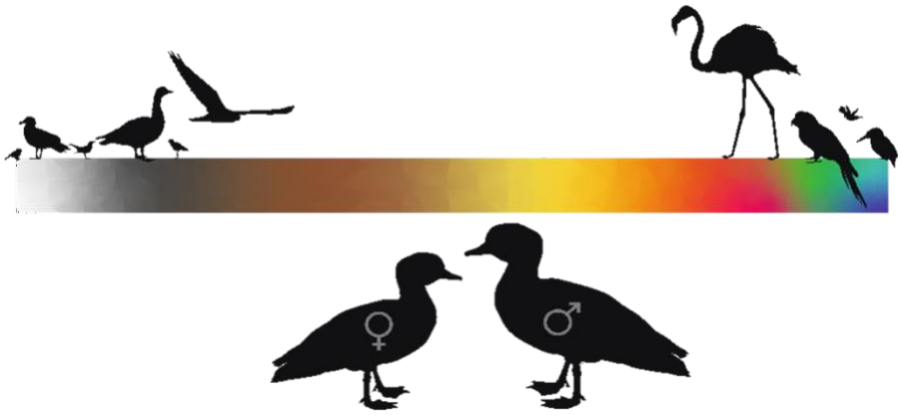


Figure 1. Sexual dimorphism in birds. Birds display a wide range of sexual dimorphism in size and plumage color.

Methods

Cancer data from managed populations of birds

To collect avian cancer records, we collaborated with numerous zoological facilities, sanctuaries, and veterinary clinics. The data represent over 25 years of pathology records from 25 different institutions using 5,499 individual necropsies,

including descriptions of age at death of 1287 individuals from 51 species, and malignancies and benign tumors across 108 bird species across 24 different avian orders managed under human care(World Association of Zoos and Aquariums, 2011). We measured malignancy prevalence and neoplasia prevalence (benign and malignant tumor) for each species by dividing the total number of necropsies reporting malignancies (or neoplasms) by the total number of necropsies available for that species (supplementary data); a measurement also used in previous studies(Boddy et al., 2020a; Kapsetaki et al., 2022).

Life history data

We assembled life-history variables from multiple published resources, including AnAge(de Magalhães & Costa, 2009) and the Amniote Life History Database(Myhrvold et al., 2015). The collected life-history variables included species averages of adult body mass (g), lifespan (months), incubation length (months), clutch size (number of offspring)(de Magalhães & Costa, 2009; Myhrvold et al., 2015), presence and degree of sexual plumage dichromatism (plumage brightness and plumage hue)(Dunn et al., 2015), and sexual size dimorphism (mass and tail size)(Lislevand et al., 2007).

Data filtering

We only included bird species for which we had at least 20 necropsies in our analysis. For analyses comparing female and male malignancy prevalence or neoplasia prevalence, as well as sex bias regressions, we used species with at least 10 necropsy records per sex. We present the neoplasia and malignancy

prevalence of 108 bird species (supplementary data). We were not able to find data on every life-history variable for every species, so in the life-history analyses, the number of species is less than 108 (body mass correlations: 100 species; lifespan correlations: 59 species; body mass x lifespan correlations: 57 species; incubation length correlations: 34 species; clutch size correlations including domesticated/semi-domesticated species: 51 species; clutch size correlations excluding domesticated/semi-domesticated species: 45 species; dimorphism in brightness correlations: 18 species; dimorphism in hue correlations: 24 species; dimorphism in mass correlations: 47 species; dimorphism in tail size correlations: 34 species; sex differences in neoplasia prevalence: 31 species). We removed all necropsies from birds in the wild. We excluded chickens (*Gallus gallus*) because as a largely domesticated agricultural species they have been selected for egg laying and frequently develop ovarian cancer(Pal et al., 2021). We excluded all infant data from our dataset because: (1) the low prevalence of age-related diseases, such as cancer, in infants would likely bias the neoplasia prevalence data towards lower values and (2) cancers in infants are medically different than adult cancers(Kattner et al., 2019). We defined infancy as a record's age that is smaller or equal to that species' age of infancy (or the average of male and female maturity). In cases of no records of infancy age, the record was considered an infant if it contained any of the following words: infant, juvenile, immature, adolescent, hatchling, subadult, neonate, newborn, offspring, fledgling. We performed correlations between clutch size and neoplasia or cancer prevalence with and without removing domesticated

and semi- domesticated species(Forshaw, 2001; Gillings et al., 2019; Leli, 1992; Orlik, 2018; Padilla-Jacobo et al., 2018; Ramírez Ayala, 2007; Q.-K. Shen et al., 2021; Svanberg, 2008; Williams, 2005; Zann & Runciman, 2003)(Supplementary data). When comparing female and male malignancy prevalence and neoplasia prevalence, we removed all cases of reproductive cancer in order to minimize any effects of controlled reproduction in managed environments on our results.

Statistical analysis

We performed all statistical analyses in R version 4.0.5(R Core Team.,2015). We prepared figures using the data visualization software ggplot2(Wickham, 2016) and performed analyses in dplyr(Wickham et al., 2018). We performed all phylogenetic analyses using the R packages ape, phytools, geiger, tidyverse, powerAnalysis (https://github.com/cran/powerAnalysis), and caper(Orme, Freckleton, Thomas, Petzoldt, Fritz, Isaac, & Pearse, 2013; Paradis & Schliep, 2019; Pennell et al., 2014; Revell, 2012b; Wickham et al., 2019) using phylogenetic generalized least squares (PGLS) regressions to take into account the phylogenetic non-independence among species(Felsenstein, 1985a) and weighting analyses by 1/(square root of the number of necropsies per species) following Revell(Revell, 2012b). We obtained avian phylogenetic trees from NCBI creator (https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi). We performed Shapiro's test(Shapiro & Wilk, 1965) to check for normality of the life history data and Grubbs' & Rosner's tests to identify and remove significant outliers. Based on the "transformTukey" function ("rcompanion" R package), which suggests a power transformation that makes values as normally distributed

as possible, we $\log_{10}$-transformed the adult body mass data, $\log_{10}$-transformed the adult mass · longevity data, transformed the longevity data to the power of 0.425, and transformed clutch size (–1 clutch size$^{-0.125}$).

We measured sexual differences in all seven biometric variables [plumage brightness, plumage hue, mass (g), and tail size (g)] as the natural log of the male biometric variable divided by the natural log of the female biometric variable. We also compared male malignancy prevalence or neoplasia prevalence versus female malignancy prevalence or neoplasia prevalence. The denominators in the case of the male malignancy prevalence or neoplasia prevalence are the total number of necropsied males, whereas the denominators in the case of the female malignancy prevalence or neoplasia prevalence are the total number of necropsied females. The distribution of the sex differences in cancer (i.e.“female malignancy prevalence minus male malignancy prevalence”, “female neoplasia prevalence minus male neoplasia prevalence”) did not follow a normal distribution and had significant outliers. Therefore, we compared malignancy prevalence and neoplasia prevalence between males and females using the non-parametric paired-samples sign test. We tested whether the *P*-values passed the False Discovery Rate (FDR) correction in each of these 26 analyses (Table 2).

Results

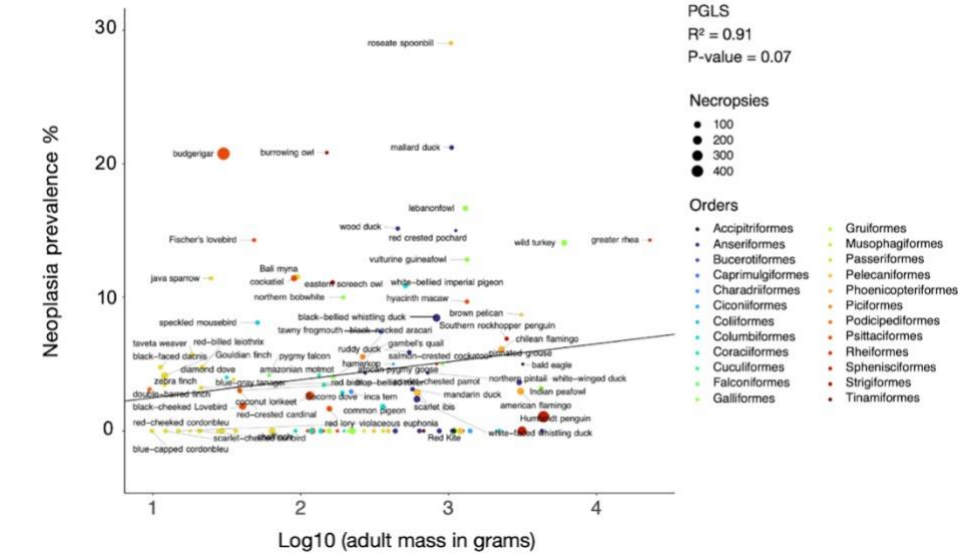
The range of neoplasia prevalence among the examined 108 bird species varied from 0% to 29%, with a mean of 4.4%, whereas malignancy prevalence among these species varied from 0% to 17.4%, with a mean of 2.3% (Table 1; supplementary data). Among the four avian taxonomic orders with at least 10

species per order in our dataset (Psittaciformes, Passeriformes, Columbiformes, and Anseriformes), the Anseriformes had on average the highest malignancy prevalence (mean ± SD: 2.84% ± 2.81%), whereas the Columbiformes had on average the lowest malignancy prevalence (mean ± SD: 1.12% ± 1.84%) (Supplementary data). We found no significant correlation between neoplasia or malignancy prevalence: and (1) adult body mass across 100 bird species and 5042 necropsies (Fig. 2A; Fig. 2B; Table 2); nor (2) adult mass times lifespan across 57 bird species and 3464 necropsies (Supp. Fig. 1A; Supp. Fig. 1B: Table 2). Neoplasia and malignancy prevalence were not higher in longer-lived birds (Fig. 3A; Fig. 3B: Table 2; 59 species and 3593 necropsies), and deaths with a necropsy diagnosis of cancer were not skewed towards old age across 1287 individuals from 51 species (Supp. Fig. 3).

We found that length of incubation was not significantly correlated with neoplasia or malignancy prevalence (Fig. 4A; Fig. 4B; Table 2; 34 species and 1806 necropsies). However, species with larger clutch sizes had significantly higher neoplasia and malignancy prevalence even after applying FDR corrections for multiple testing (*P*-value = 0.005, R^2 = 0.99; and *P*-value = 0.0019, R^2 = 0.99, respectively; Fig. 5; 51 species and 2119 necropsies), and after controlling for species body mass (*P*-value = 0.005, R^2 = 0.17; and *P*-value =0.0014, R^2 = 0.17, respectively; Table 2). The positive correlation between clutch size and malignancy prevalence, but not neoplasia prevalence, remained significant after removing domesticated and semi-domesticated species (*P*-value = 0.004, R^2 = 0.99; Supp. Fig. 4B; 45 species and 1839 necropsies) and

controlling for body mass (*P*-value = 0.004, R^2 = 0.1; Table 2; 45 species). We found no significant associations between neoplasia or malignancy prevalence and several sexually dimorphic and dichromatic traits (Fig. 6; Table 2). Also, neoplasia and malignancy prevalence were not significantly different between males and females across 31 species (Fig. 7; Supp. Fig. 2; Table 2).

A



B

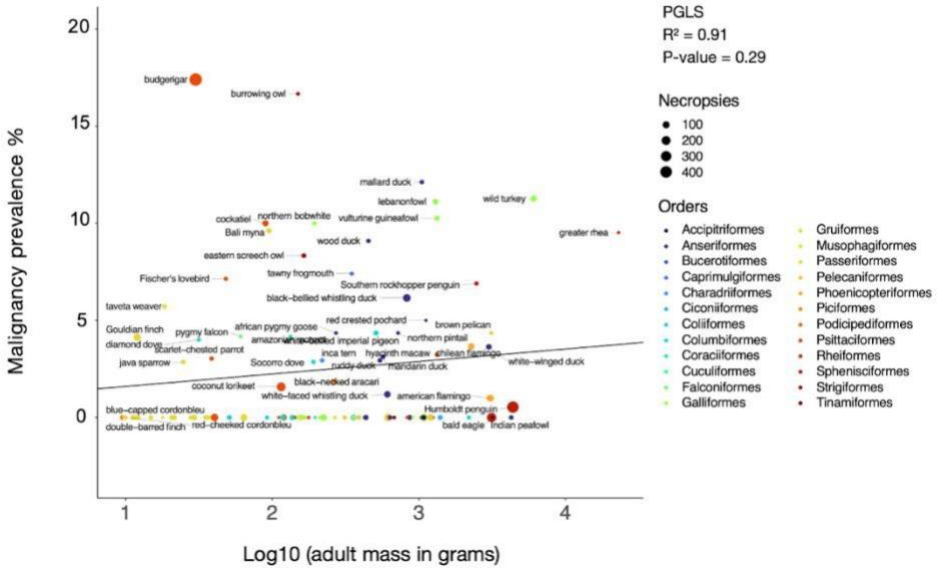
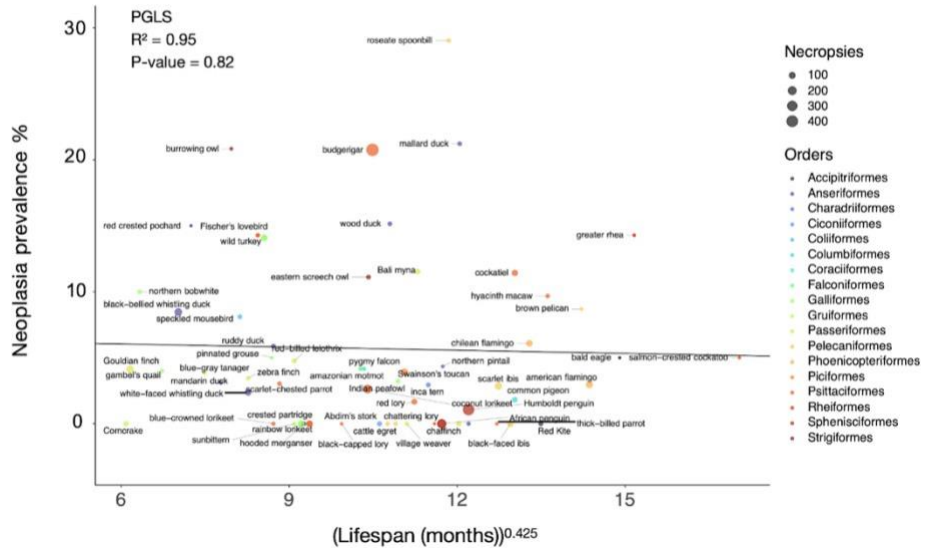


Figure 2. Larger body mass is not correlated with neoplasia prevalence (A) or malignancy prevalence (B) across 100 bird species. Dot size indicates the number of necropsies per species. Colors show the taxonomic order of each species, and black lines show the phylogenetically-controlled linear regression of the logarithm of adult mass versus malignancy prevalence or neoplasia prevalence.

A



B

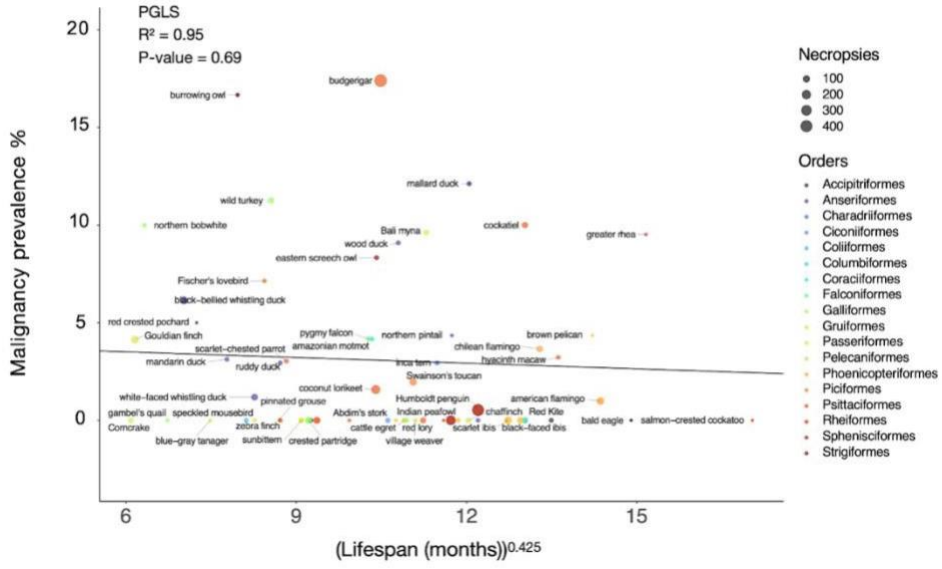
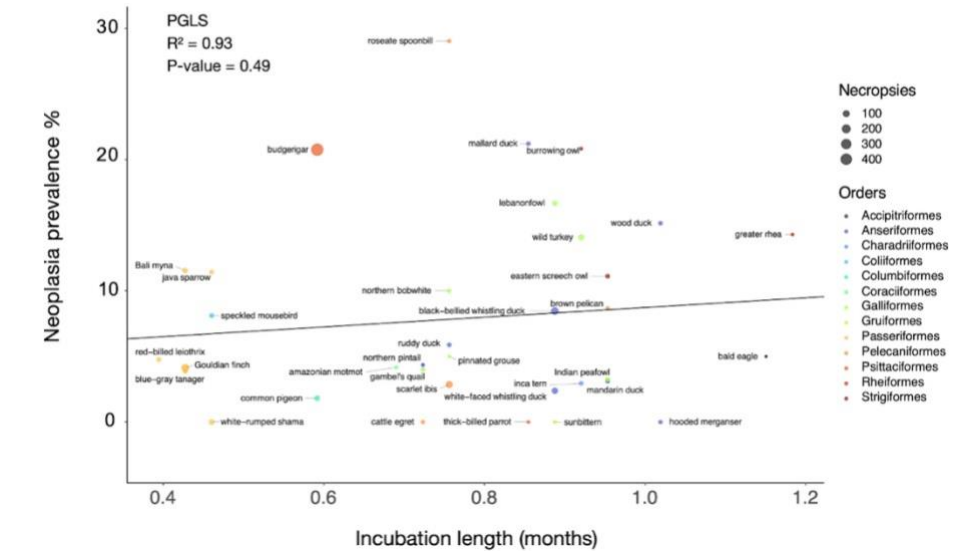


Figure 3. Longer lifespan is not correlated with neoplasia prevalence (A) or malignancy prevalence (B) across 59 bird species. Dot size indicates the number of necropsies per species. Colors show the taxonomic order of each species. Black lines show the phylogenetically-controlled linear regression of the normalized values of species lifespan versus malignancy prevalence or neoplasia prevalence.

A



B

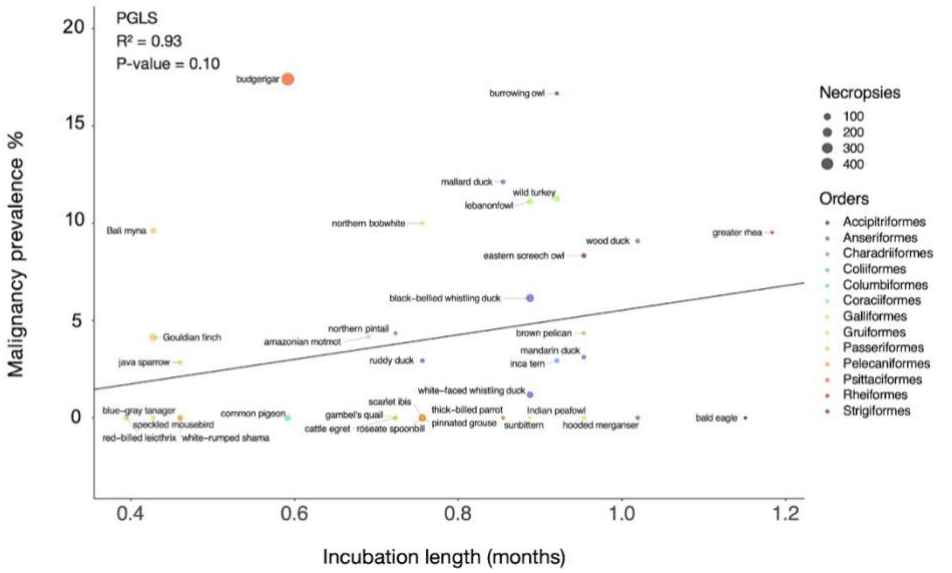
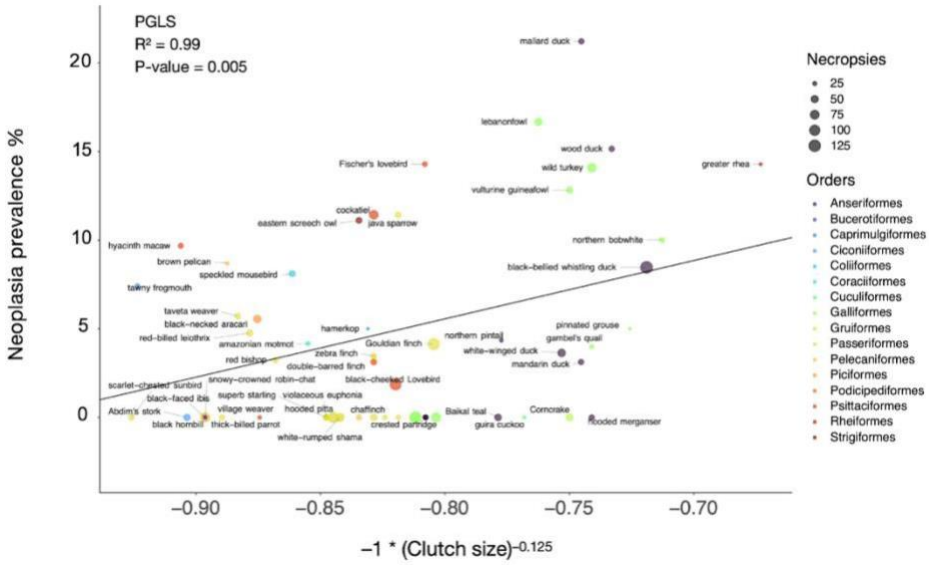


Figure 4. Incubation length is not correlated with neoplasia prevalence (A) or

malignancy prevalence (B) when controlling for body mass across 34 bird species. Different colors indicate the order in which each species belongs and the size of the dot indicates the number of necropsies per species. Black lines show the phylogenetically-controlled linear regression of incubation length versus malignancy prevalence or neoplasia prevalence.

A



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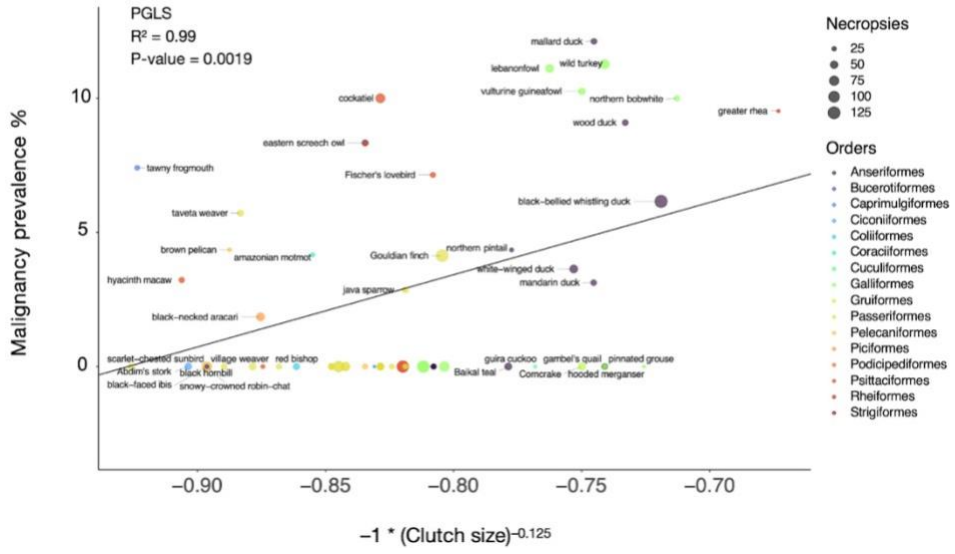
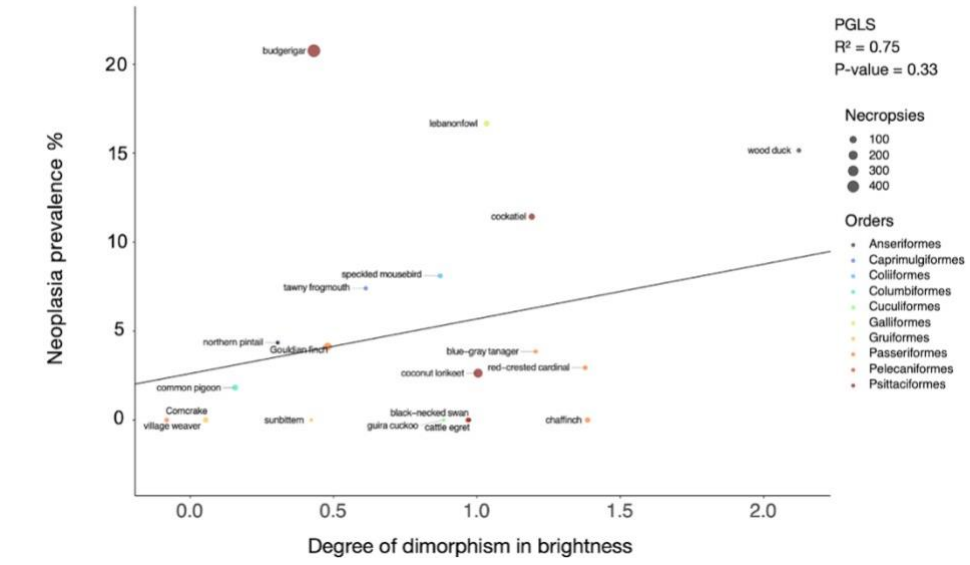
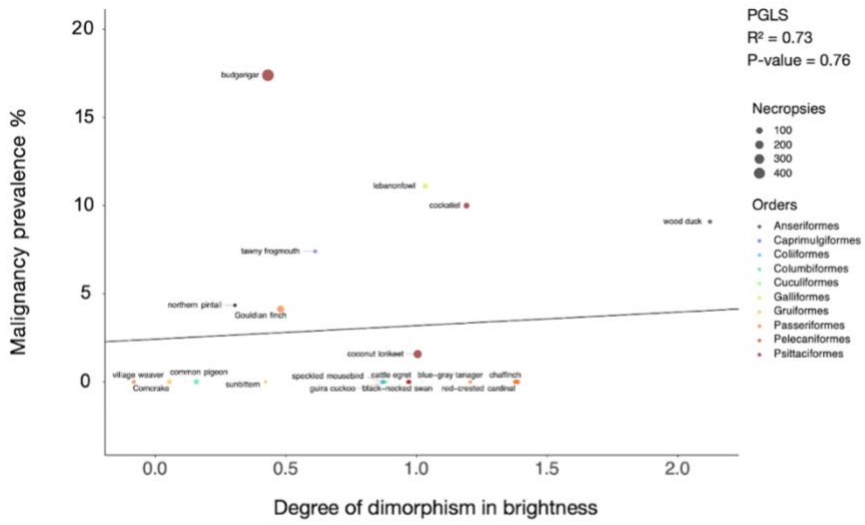


Figure 5. Larger clutch size is correlated with neoplasia prevalence (A) and malignancy prevalence (B) across 51 bird species. The -1 * (Clutch size)$^{-0.125}$ function was suggested by the Tukey transform to make the clutch size data into a normal distribution. After controlling for species body mass, the positive correlation between clutch size and neoplasia prevalence (*P*-value = 0.005; Table 2) and malignancy prevalence (*P*-value = 0.0014; Table 2) remains significant. Dot size indicates the number of necropsies per species. Colors show the taxonomic order of each species. Black lines show the phylogenetically controlled linear regression of the normalized values of clutch size versus malignancy prevalence or neoplasia prevalence.

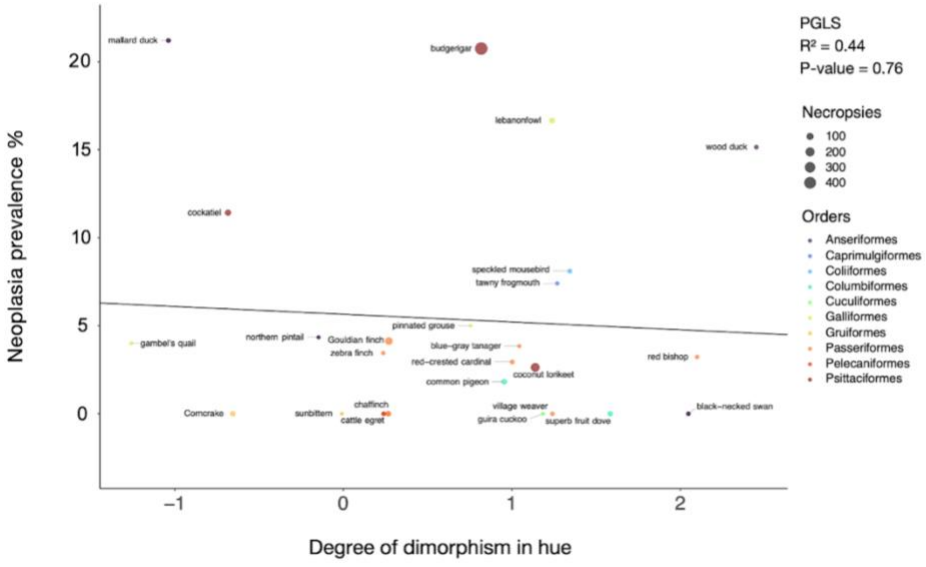
A



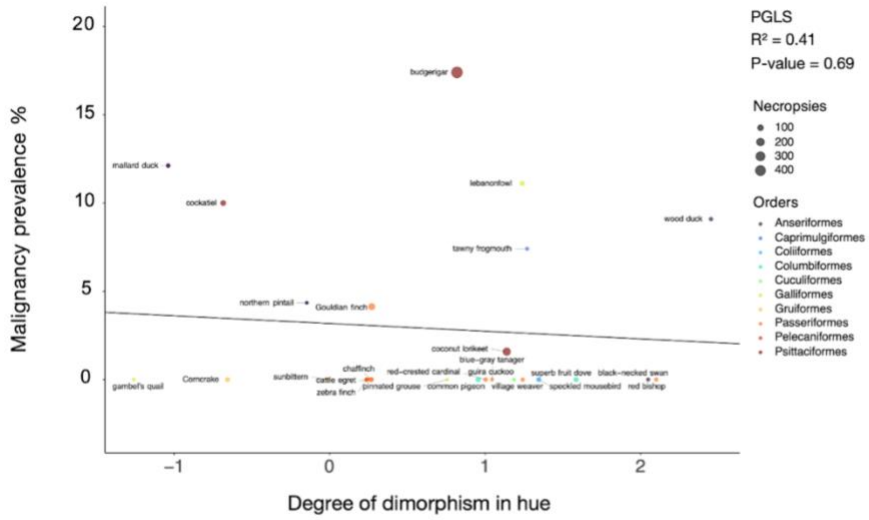
B


Malignancy prevalence %
Degree of dimorphism in brightness
PGLS
R² = 0.73
P-value = 0.76
Necropsies
100
200
300
400
Orders
Anseriformes
Caprimulgiformes
Coliiformes
Columbiformes
Cuculiformes
Galliformes
Gruiformes
Passeriformes
Pelecaniformes
Psittaciformes
budgerigar
lebanonfowl
cockatiel
wood duck
tawny frogmouth
northern pintail
Gouldian finch
coconut lorikeet
village weaver
common pigeon
Comorako
sunbittern
speckled mousebird
cattle egret
blue-gray tanager
chaffinch
guira cuckoo
black-necked swan
red-crested cardinal

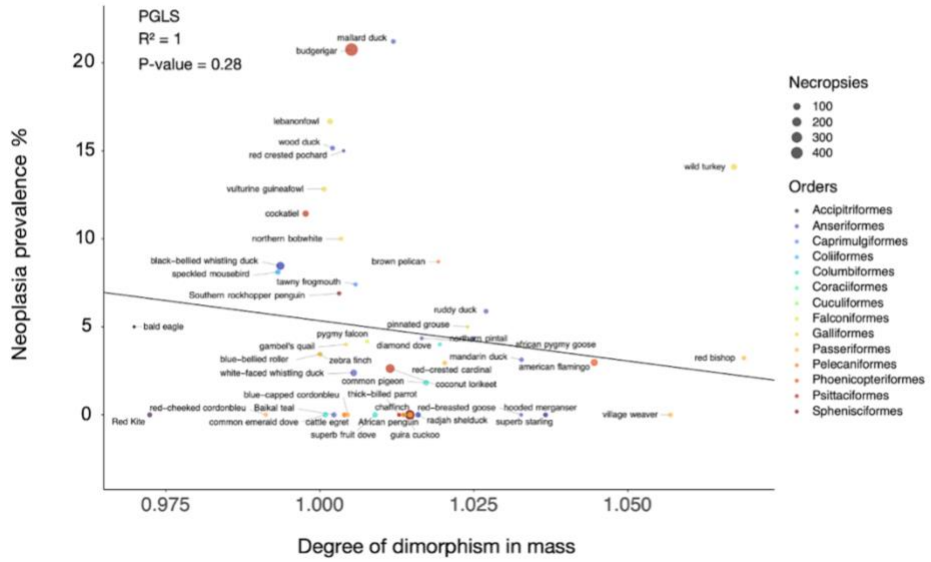
C


Neoplasia prevalence %
Degree of dimorphism in hue
PGLS
R² = 0.44
P-value = 0.76
Necropsies
100
200
300
400
Orders
Anseriformes
Caprimulgiformes
Coliiformes
Columbiformes
Cuculiformes
Galliformes
Gruiformes
Passeriformes
Pelecaniformes
Psittaciformes
mallard duck
budgerigar
lebanonfowl
wood duck
cockatiel
speckled mousebird
tawny frogmouth
pinnated grouse
gambel's quail
northern pintail
Gouldian finch
zebra finch
blue-gray tanager
red-crested cardinal
coconut lorikeet
red bishop
common pigeon
chaffinch
Comorako
sunbittern
cattle egret
village weaver
guira cuckoo
superb fruit dove
black-necked swan

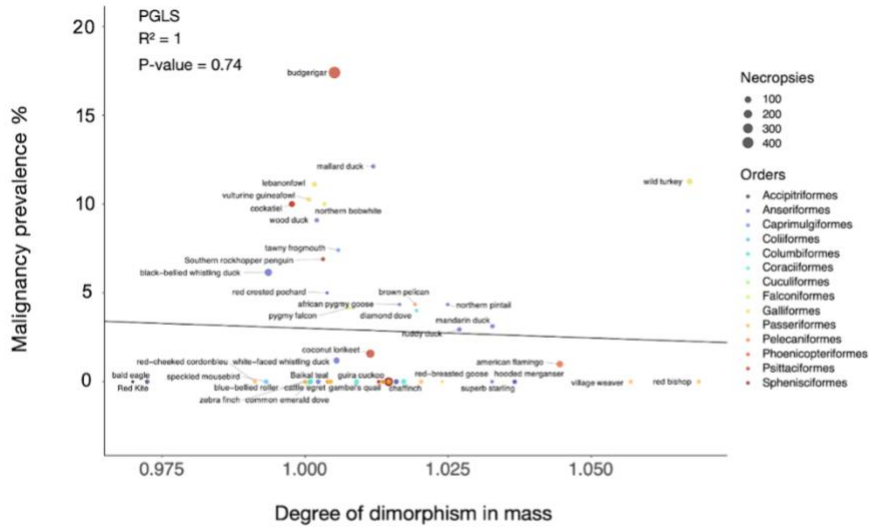
D


PGLS
R² = 0.41
P-value = 0.69
Necropsies
100
200
300
400
Orders
Anseriformes
Caprimulgiformes
Coliiformes
Columbiformes
Cuculiformes
Galliformes
Gruiformes
Passeriformes
Pelecaniformes
Psittaciformes
Malignancy prevalence %
Degree of dimorphism in hue
budgerigar
mallard duck
lebanonfowl
cockatiel
wood duck
tawny frogmouth
northern pintail
Gouldian finch
coconut lorikeet
blue-gray tanager
chaffinch
red-crested cardinal
guira cuckoo
superb fruit dove
black-necked swan
gambel's quail
Corncrake
sunbittern
cattle egret
zebra finch
pinnated grouse
common pigeon
village weaver
speckled mousebird
red bishop

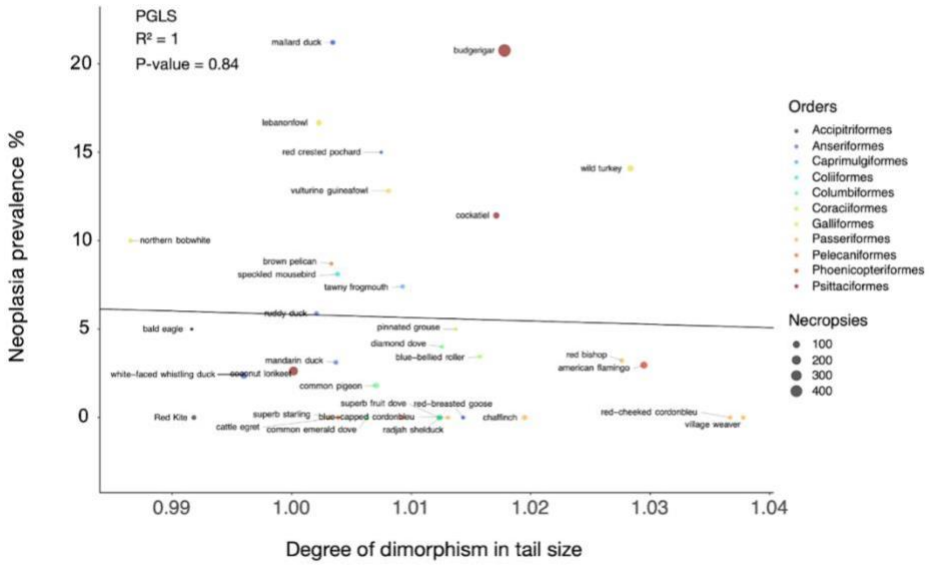
E


PGLS
R² = 1
P-value = 0.28
Necropsies
100
200
300
400
Orders
Accipitriformes
Anseriformes
Caprimulgiformes
Coliiformes
Columbiformes
Coraciiformes
Cuculiformes
Falconiformes
Galliformes
Passeriformes
Pelecaniformes
Phoenicopteriformes
Psittaciformes
Spheniscifformes
Neoplasia prevalence %
Degree of dimorphism in mass
mallard duck
budgerigar
lebanonfowl
wood duck
red crested pochard
wild turkey
vulturine guineafowl
cockatiel
northern bobwhite
black-bellied whistling duck
speckled mousebird
brown pelican
tawny frogmouth
Southern rockhopper penguin
ruddy duck
pinnated grouse
bald eagle
pygmy falcon
northern pintail
gambel's quail
diamond dove
african pygmy goose
blue-bellied roller
zebra finch
mandarin duck
red bishop
white-faced whistling duck
red-crested cardinal
american flamingo
common pigeon
coconut lorikeet
blue-capped cordonbleu
thick-billed parrot
red-cheeked cordonbleu
Baikal teal
chaffinch
red-breasted goose
hooded merganser
village weaver
Red Kite
common emerald dove
cattle egret
African penguin
radjah shelduck
superb starling
superb fruit dove
guira cuckoo

F


PGLS
R² = 1
P-value = 0.74
Malignancy prevalence %
Degree of dimorphism in mass
0.975
1.000
1.025
1.050
0
5
10
15
20
budgerigar
mallard duck
wild turkey
lebanonfowl
vulturine guineafowl
cockatiel
northern bobwhite
wood duck
tawny frogmouth
Southern rockhopper penguin
black-bellied whistling duck
red crested pochard
brown pelican
african pygmy goose
northern pintail
pygmy falcon
diamond dove
mandarin duck
ruddy duck
coconut lorikeet
red-cheeked cordonbleu
white-faced whistling duck
american flamingo
bald eagle
Red Kite
speckled mousebird
Baikal teal
guina cuckoo
red-breasted goose
hooded merganser
blue-bellied roller
cattle egret
gambel's quail
chaffinch
superb starling
village weaver
red bishop
zebra finch
common emerald dove
Necropsies
100
200
300
400
Orders
Accipitriformes
Anseriformes
Caprimulgiformes
Coliiformes
Columbiformes
Coraciiformes
Cuculiformes
Falconiformes
Galliformes
Passeriformes
Pelecaniformes
Phoenicopteriformes
Psittaciformes
Sphenisciformes

G


PGLS
R² = 1
P-value = 0.84
Neoplasia prevalence %
Degree of dimorphism in tail size
0.99
1.00
1.01
1.02
1.03
1.04
0
5
10
15
20
mallard duck
budgerigar
lebanonfowl
red crested pochard
wild turkey
vulturine guineafowl
cockatiel
northern bobwhite
brown pelican
speckled mousebird
tawny frogmouth
ruddy duck
bald eagle
pinnated grouse
diamond dove
blue-bellied roller
red bishop
american flamingo
mandarin duck
white-faced whistling duck
coconut lorikeet
common pigeon
superb fruit dove
red-breasted goose
Red Kite
superb starling
blue-capped cordonbleu
chaffinch
red-cheeked cordonbleu
village weaver
cattle egret
common emerald dove
radjah shelduck
Orders
Accipitriformes
Anseriformes
Caprimulgiformes
Coliiformes
Columbiformes
Coraciiformes
Galliformes
Passeriformes
Pelecaniformes
Phoenicopteriformes
Psittaciformes
Necropsies
100
200
300
400

H

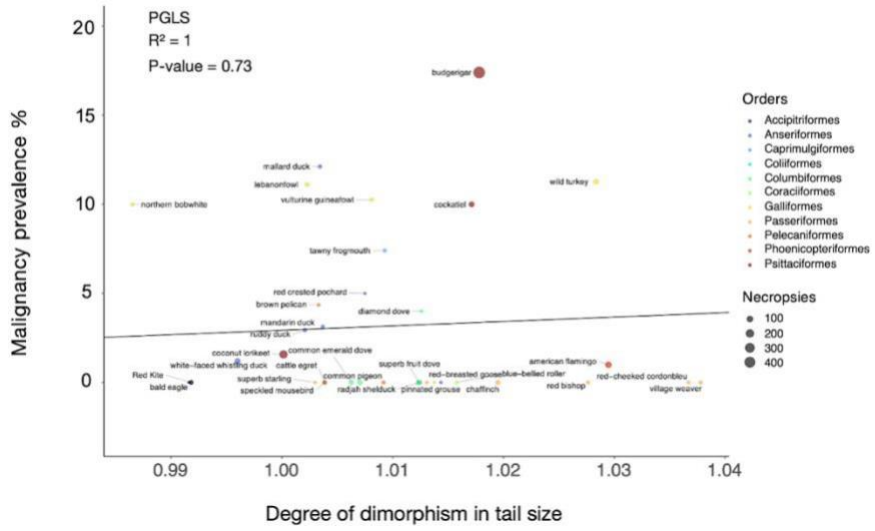


Figure 6. Sexual dimorphic traits are not correlated with neoplasia or malignancy prevalence in birds. The degree of dimorphism in brightness is not correlated with neoplasia prevalence (A) or malignancy prevalence (B) across 18 species of birds. The degree of dimorphism in hue is not correlated with neoplasia prevalence (C) or malignancy prevalence (D) across 24 species of birds. The degree of dimorphism in mass is not correlated with neoplasia prevalence (E) or malignancy prevalence (F) across 47 species of birds. The degree of dimorphism in tail size is not correlated with neoplasia prevalence (G) or malignancy prevalence (H) across 34 species of birds. A positive score on the x-axis indicates that the species has a relatively higher score in that trait in males than females, whereas a negative score on the x-axis shows that the species has a relatively higher score in that trait in females than males. Black lines show the phylogenetically-controlled linear regression of the degree of dimorphism in the trait versus neoplasia prevalence or malignancy prevalence. Different colors indicate the order in which each species belongs and the size of the dot indicates the total number of necropsies per species.

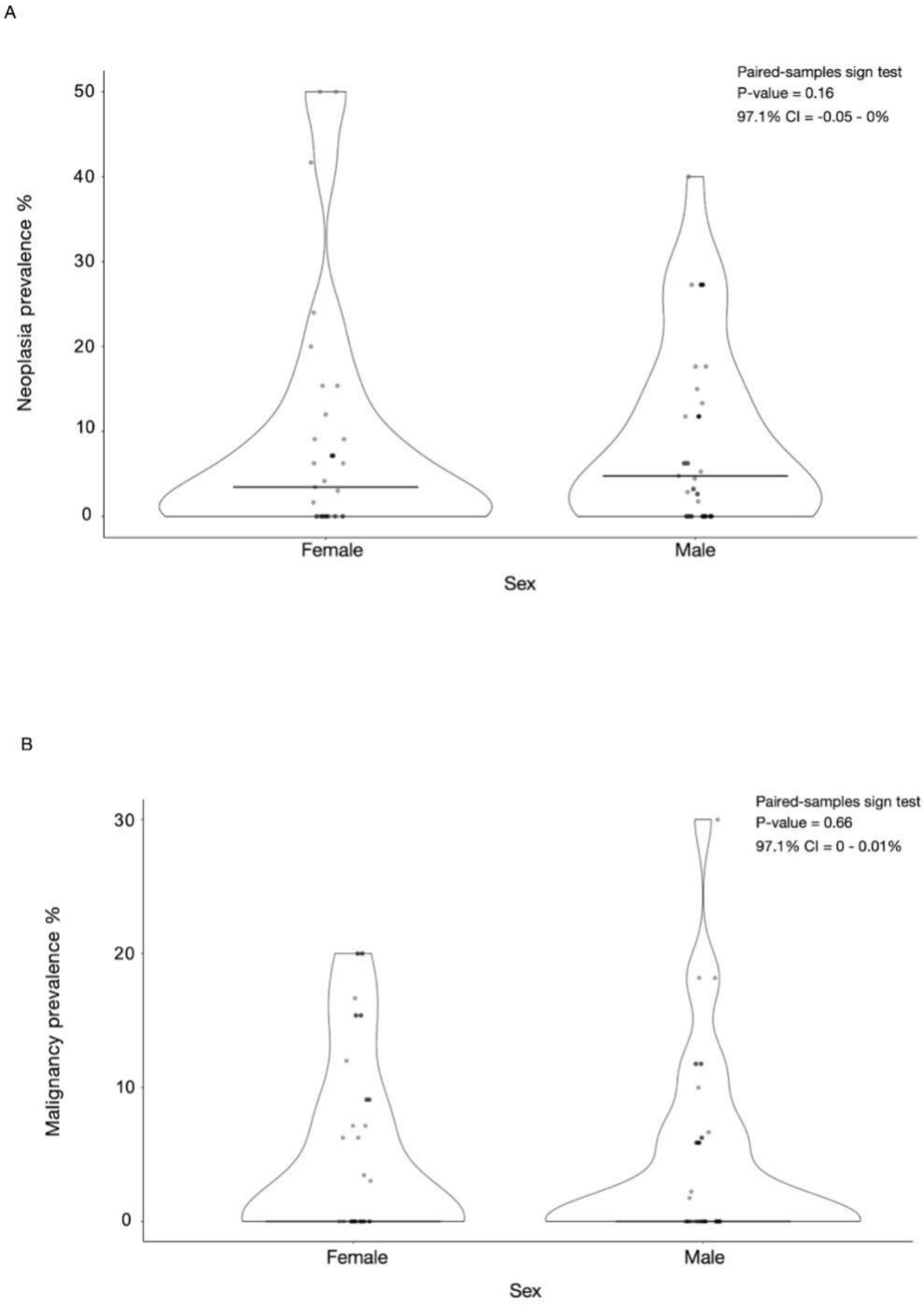


Figure 7. Neoplasia (A) and malignancy prevalence (B) are not significantly different between females and males across 31 bird species. Horizontal bars show the median neoplasia (A) or malignancy prevalence (B). We added minimal

jitter for better visualization of individual data points.

Tables

Table 1. Species (A, B) with the highest and lowest malignancy prevalence and neoplasia prevalence.

A. Species with the highest neoplasia prevalence and lowest malignancy prevalence. This table includes 10 species with the highest neoplasia prevalence and lowest malignancy prevalence in our dataset (supplementary data). Another 54 species in our dataset have 0% malignancy prevalence (Supplementary data).

Species (common name)	**↑ Neoplasia prevalence (necropsies)**	**Species (common name)**	**↓ Malignancy prevalence (necropsies)**
Platalea ajaja (roseate spoonbill)	29.03% (31)	*Spheniscus* demersus (African penguin)	0% (210)
Ardeola speciosa (javan pond heron)	21.4% (42)	*Lophura edwardsi* (Edwards's pheasant)	0% (110)
Anas platyrhynchos (mallard duck)	21.2% (33)	*Agapornis nigrigenis* (black-cheeked lovebird)	0% (108)
Melopsittacus undulatus (budgerigar)	20.7% (477)	*Eudocimus ruber* (scarlet ibis)	0% (105)
Athene cunicularia (burrowing owl)	20.8% (24)	*Pitta sordida* (hooded pitta)	0% (89)
Numida meleagris (lebanonfowl)	16.6% (54)	*Rollulus rouloul* (crested partridge)	0% (80)
Aix sponsa (wood duck)	15.1% (33)	*Trichoglossus moluccanus* (rainbow lorikeet)	0% (80)

Netta rufina (red crested pochard)	15% (20)	*Theristicus melanopis* (black-faced ibis)	0% (72)
Rhea americana (greater rhea)	14.2% (21)	*Eos bornea* (red lory)	0% (60)
Agapornis fischeri (Fischer's lovebird)	14.2% (28)	*Copsychus malabaricus* (white-rumped shama)	0% (59)

B. Species with the highest malignancy prevalence and lowest neoplasia prevalence. This table includes 10 species with the highest malignancy prevalence and lowest neoplasia prevalence in our dataset (supplementary data). Another 34 species in our dataset have 0% neoplasia prevalence (Supplementary data).

Species (common name)	**↑ Malignancy prevalence (necropsies)**	**Species (common name)**	**↓ Neoplasia prevalence (necropsies)**
Melopsittacus undulatus (budgerigar)	17.4% (477)	*Spheniscus demersus* (African penguin)	0% (210)
Athene cunicularia (burrowing owl)	16.6% (24)	*Lophura edwardsi* (Edwards's pheasant)	0% (110)
Anas platyrhynchos (mallard duck)	12.1% (33)	*Pitta sordida* (hooded pitta)	0% (89)
Meleagris gallopavo (wild turkey)	11.2% (71)	*Rollulus rouloul* (crested partridge)	0% (80)
Numida meleagris (lebanonfowl)	11.1% (54)	*Trichoglossus moluccanus* (rainbow lorikeet)	0% (80)
Acryllium vulturinum (vulturine guineafowl)	10.2% (39)	*Theristicus melanopis* (black-faced ibis)	0% (72)

Nymphicus hollandicus (cockatiel)	10% (70)	*Copsychus malabaricus* (white-rumped shama)	0% (59)
Colinus virginianus (northern bobwhite)	10% (30)	*Chalcophaps indica* (common emerald dove)	0% (48)
Leucopsar rothschildi (Bali myna)	9.6% (52)	*Ptilinopus superbus* (superb fruit dove)	0% (48)
Rhea americana (greater rhea)	9.5% (21)	*Crex crex* (corncrake)	0% (47)

Table 2. Summary statistics. We present the summary statistics of phylogenetic regressions (PGLS) between neoplasia and malignancy prevalence and life history variables, except for the comparison of neoplasia and malignancy prevalence in females and males for which we present the summary statistics of paired-samples sign tests. The number of species analyzed is different in the majority of analyses. This is due to the fact that not all life history variables are available for every species in the literature. In the 1st P-value column we report the *P*-value of the first variable (i.e., variable A in the multivariate analysis), and in the 2nd *P*-value column we report the *P*-value of variable B. We highlight the *P*-values that passed the False Discovery Rate (FDR) correction with an asterisk (*). In the F-statistics column we report the F-statistics of variable A, and in the "Type of Association" column we report the positive (+) or negative (–) correlation between the variable A and the prevalence of neoplasia or malignancy. High lambda values show that the associations are mainly explained by common ancestry. † indicates that the R^2 value was not available.

Independent variable(s)	Figure	Dependent variable	R^2	F-statistic and degrees of freedom (DF)	Lambda	Type of association	P-value of variable A	P-value of variable B
log10 adult mass	2A	Neoplasia prevalence	0.91	3.19 on 1 and 98 DF	0.00006	+	0.07	NA†
	2B	Malignancy prevalence		0.91 1.09 on 1 and 98 DF	0.22	+	0.29	NA†
$lifespan^{0.425}$	3A	Neoplasia prevalence		0.95 0.04 on 1 and 57 DF	0.00006	–	0.82	NA†
	3B	Malignancy prevalence		0.95 0.15 on 1 and 57 DF	0.00006	–	0.69	NA†
incubation length	4A	Neoplasia prevalence		0.93 0.47 on 1 and 32 DF	0.00006	+	0.49	NA†
	4B	Malignancy prevalence		0.93 2.73 on 1 and 32 DF	0.00006	+	0.10	NA†
$-1 \cdot clutch\ size^{-0.125}$	5A	Neoplasia prevalence		0.99 8.31 on 1 and 49 DF	0.00006	+	0.005*	NA†
	5B	Malignancy prevalence		0.99 10.80 on 1 and 49 DF	0.01	+	0.0019*	NA†
$-1 \cdot clutch\ size^{-0.125}$ + log10 adult mass		Neoplasia prevalence		0.17 8.38 on 1 and 48 DF	0.00006	+	0.005*	0.19
		Malignancy prevalence		0.17 11.48 on 1 and 48 DF	0.00006	+	0.0014*	0.05
$-1 \cdot clutch\ size^{-0.125}$ (having excluded domesticated and semi-domesticated species)	Supp. Fig. 4A	Neoplasia prevalence		0.99 3.68 on 1 and 43 DF	0.009	+	0.06	NA†
	Supp. Fig. 4B	Malignancy prevalence		0.99 8.78 on 1 and 43 DF	0.00006	+	0.004*	NA†

$-1 \cdot \text{clutch size}^{-0.125} + \log10$ adult mass (having excluded domesticated and semi-domesticated species)		Neoplasia prevalence	0.08	3.91 on 1 and 42 DF	0.00006	+	0.05	0.35
		Malignancy prevalence	0.1	8.9 on 1 and 42 DF	0.00006	+	0.004*	0.21
degree of dimorphism in brightness	6A	Neoplasia prevalence	0.75	1.00 on 1 and 16 DF	0.11	+	0.33	NA†
	6B	Malignancy prevalence	0.73	0.09 on 1 and 16 DF	0.33	+	0.76	NA†
degree of dimorphism in hue	6C	Neoplasia prevalence	0.44	0.09 on 1 and 22 DF	0.10	–	0.76	NA†
	6D	Malignancy prevalence	0.41	0.15 on 1 and 22 DF	0.35	–	0.69	NA†
degree of dimorphism in mass	6E	Neoplasia prevalence	1	1.14 on 1 and 45 DF	0.24	–	0.28	NA†
	6F	Malignancy prevalence	1	0.10 on 1 and 45 DF	0.32	–	0.74	NA†
degree of dimorphism in tail size	6G	Neoplasia prevalence	1	0.03 on 1 and 32 DF	0.40	–	0.84	NA†
	6H	Malignancy prevalence	1	0.11 on 1 and 32 DF	0.60	+	0.73	NA†
sex	7A	Neoplasia prevalence	97.1% CI = -0.05 - 0%				0.16	NA†

	7B	Malignancy prevalence	97.1% CI = 0 - 0.01%		0.66_{NA}†
log10 (adult mass · lifespan)	Supp. Fig. 1A	Neoplasia prevalence	0.970.06 on 1 and 55 DF	0.00006+	0.79_{NA}†
	Supp. Fig. 1B	Malignancy prevalence	0.970.001 on 1 and 55 DF	0.00006–	0.97_{NA}†

Discussion

We hypothesized that differences in life-history traits, including clutch size, may explain some of the variation in cancer prevalence across managed bird species. Species varied in their clutch sizes from scarlet-chested sunbirds laying on average 1.85 eggs, to greater rheas laying >10 times as many (23 eggs on average). We found that clutch size explained a statistically significant portion (17%) of the variation in cancer prevalence when controlling for $\log_{10}$ adult mass. Species with larger clutch size had higher malignancy and neoplasia prevalence, even after FDR corrections and controlling for body mass. The positive correlation between clutch size and malignancy prevalence remained significant even after removing domesticated and semi-domesticated species from the analysis. However, no other life-history trait that we measured, such as adult body mass, lifespan, incubation length, sexual size dimorphism or sexual dichromatism, explained the variance in avian cancer prevalence, nor was there a significant difference in cancer or neoplasia prevalence between male and female birds.

Body mass and lifespan are not associated with cancer in birds under human care

Our observations in populations of birds managed under human care show no significant correlation between neoplasia or malignancy prevalence and adult body mass, lifespan, or adult mass times lifespan in birds, supporting Peto's paradox(Peto et al., 1975); however, these results are in contrast to the observation of cancer in free-living birds(Møller et al., 2017). While there is a trend in our data for larger birds to have more cancer, this was not statistically significant (*P*-value = 0.29). If there is a real discrepancy between our study and that of Møller et al.(Møller et al., 2017), it may be due to the different number of individuals sampled per species (≥3 records per species in Møller et al.(Møller et al., 2017) versus ≥20 necropsies per species in our study), the different species of birds analyzed (238 free-living bird species in Denmark(Møller et al., 2017) versus 108 managed bird species from multiple institutions), or body mass mostly measured with a precision balance(Møller et al., 2017) versus collected from the literature. In addition, birds collected by Mølller et al. were mostly killed by hunters (both human and non-human), whereas those in our study were protected from predation and thus allowed to live long enough to succumb to various diseases of old age, including cancer. Unfortunately, only six species of birds are included in both Møller et al.(Møller et al., 2017) and this study, limiting our ability to compare cancer prevalence in wild versus managed birds. In general, patterns of tumor incidence or neoplasia prevalence were consistent between these free-living birds and populations managed under human care

(Supplementary Table 1). Therefore, while there are many potential sources of error in the enumeration of the life-history traits and neoplasia prevalence in either wild or managed birds, it is promising that there is consistency in shared data trends across studies.

Interestingly, the roseate spoonbill, ranked 18th in the order of longest to shortest living species among the 59 bird species with lifespan data in our dataset, has the highest neoplasia prevalence (29.03%), but no reported malignancy (0% malignancy prevalence). We found that birds that live longer do not have significantly higher cancer prevalence than shorter-lived species, and there is not a skew in terms of more cancer deaths towards old age. This may be explained by the observation that long-lived birds have coevolved pathways that increase longevity in part through decreasing cancer rates(Roche et al., 2012; Wirthlin et al., 2018). Specifically, in long-lived birds, there is an increased selective pressure for genes related to controlling cell division and tumor suppression(Wirthlin et al., 2018). Long-lived mammals, such as bats, have extra copies of *FBXO31* and mutations in the insulin-like growth factor 1 receptor/growth-hormone receptor related to blocking the cell cycle and responding to DNA damage(Santra et al., 2009; Seim et al., 2013; Seluanov et al., 2018). The fact that erythrocyte telomeres of long-lived birds shorten at a slower pace than erythrocyte telomeres of shorter-lived birds(Haussmann et al., 2003) may provide an additional mechanistic explanation for the lower than expected cancer prevalence in long-lived birds.

Neoplasia and cancer prevalences are higher in species with larger clutch

sizes

Our results are consistent with previous findings that larger litter size is associated with cancer prevalence in mammals(Abegglen et al., 2015; Boddy et al., 2020a). Many of the life-history traits described in this article, such as body mass, number of offspring produced, incubation time, and longevity, are tightly linked with each other(Charnov, 1993; Clutton-Brock, 1991; Kihlström, 1972; Stearns, 1992; West et al., 2000) (Supp. Fig. 5). No significant correlations were found between cancer prevalence and lifespan, adult mass, or incubation/gestation length in birds or mammals(Boddy et al., 2020a). Larger clutch size is correlated with malignancy prevalence and neoplasia prevalence, even after corrections for multiple testing and controlling for species body mass. This discrepancy between clutch size predicting neoplasia prevalence but not the other (correlated) life-history variables may be due to the fact that we only have clutch size data on a subset of the species (51 bird species) for which we have other life-history data (e.g. 100 bird species with adult mass data). It could also be that distinct molecular pathways associated with clutch size have coevolved with increased neoplasia and malignancy prevalence.

We found that when including domesticated species in the analyses, both malignancy and neoplasia prevalence are positively correlated with clutch size, however, when excluding domesticated species, only malignancy prevalence remains positively correlated with clutch size; indicating that differential selection pressures may be acting on neoplasia versus malignancy. While most aviariesuse natural light, we speculate that the exposure to artificial light could be one explanation for the association between neoplasia prevalence and clutch

size when domesticated species are included in the analysis. Artificial light is used in poultry industries, as well as parakeet breeding, to lengthen the hours of egg laying(Schlumberger, 1954; Staffe, 1951), and such prolonged exposure to light of high intensity has been suggested to cause hyperplasia and neoplasia in the pituitary(Schlumberger, 1954).

Is sexual dimorphism or dichromatism correlated with cancer prevalence in birds?

The strength of sexual selection could impose energetic constraints resulting in tradeoffs between investment in mate competition and somatic (anti-cancer) maintenance(Boddy et al., 2015). Sexually dimorphic or dichromatic species with extreme phenotypes, such as large and colorful ornaments or weapons, may have an increased risk of cancer(Boddy et al., 2015). This may be because selection for rapid cell growth in these tissues leads to the potential increased tumor growth as a byproduct. It may also be that there is selection for increased allocation of resources towards these costly sexual traits(Doutrelant et al., 2012; Kemp et al., 2011) at the expense of DNA repair and immune defenses(Boddy et al., 2015). However, even though testosterone in male red-legged partridges can increase the concentration of carotenoids, responsible for colorful traits, and testosterone suppresses the immune system, carotenoids also have immunoenhancing effects(Blas et al., 2006). We found no significant difference in cancer prevalence in relation to sexual dimorphism and dichromatism. When factoring in both hue (the dominant wavelength of color) and brightness (the intensity of color), the degree of sexual dichromatism showed no significant correlation with neoplasia or malignancy prevalence. While most males tend to be larger than the females, that is not always the case, especially within birds of prey(Amadon, 1975). When examining the degree of sexual size dimorphism, we found no significant difference in cancer prevalence and differing sizes between sexes. This means that sexually dimorphic birds who spend time and energy in creating colorful plumage or larger body parts do not seem to pay

a cost in terms of cancer susceptibility. It is possible that the birds in our study did not experience such tradeoffs because in captivity they may have high energy budgets that allow them to invest both in sexually selected traits as well as in somatic maintenance in the form of cancer suppression. The same might not be the case for wild birds who are under greater energetic constraints and might therefore be more likely to experience tradeoffs.

Do female birds have higher cancer prevalence than male birds?

Cancer rates in most other species, including humans, are biased toward males(Dorak & Karpuzoglu, 2012). Current theory states that the double X chromosome found in females may offer some cancer protection(Dorak & Karpuzoglu, 2012). For example, if the X chromosome carries a cancer-inducing mutation, the extra X chromosome present in females may carry a non-deleterious variant of the allele, whereas males (XY) without the extra X chromosome would not have this protective variant. In alignment with the two-X chromosome theory of cancer protection, previous work has shown that female birds (ZW) have more neoplasms than male birds (ZZ), but this was not validated statistically with sex-specific neoplasia prevalence(Effron et al., 1977a). We found that females do not have significantly different neoplasia prevalence or malignant prevalence than male birds. This analysis was done excluding reproductive cancers because living in managed environments with controlled reproduction could be affecting the animals' susceptibility to cancers of the reproductive system.

Future directions

We constructed a large and high-quality dataset including not only a significantly larger number of life history variables for birds than previous studies, but also detailed necropsy information for a large number of individuals per species, allowing greater error reduction, the inclusion of potential covariant traits, as well as the ability to distinguish benign and malignant tumors. Still, our study does not have information about the exact tissue where neoplasms were found in every individual, and future studies would benefit from knowledge of the relationships between distinct cancer types and life history in birds. There may also be evolutionary mismatches between animals in zoological institutions and in the wild. For example, 84% of the mammalian species analyzed by Tidière et al.(Tidière et al., 2016) lived longer in zoos than in the wild. Future studies using a larger dataset with tracked life history and cancer records for every individual and tissue from birds in zoological institutions and in the wild would be helpful to better understand the role of life-history traits in cancer susceptibility.

Recent studies have focused on the evolutionary history of specific oncogenes in birds(Opazo et al., 2021). Specifically, the expansion of an oncoprotein, Golgi phosphoprotein 3, may contribute to birds' relatively lower cancer susceptibility(Opazo et al., 2021) compared to mammals(Effron et al.,1977a; Madsen et al., 2017b; Pesavento et al., 2018). Although Golgi phosphoprotein 3 has many functions, such as modulating the dynamics of adhesion(Arriagada et al., 2019) and regulating the function of mitochondria(Nakashima-Kamimura et al., 2005), its exact molecular association

with cancer suppression is not entirely clear(Opazo et al., 2021). Future work could examine the possible variation in the number of oncogenes and tumor-suppressors across bird species to identify how they are linked with cancer susceptibility and large clutch/litter size, and whether this correlation occurs in wild animals or is an artifact of domestication and artificial selection.

Several ecological factors may be driving many of the cancers in birds in our dataset. Previous work in chickens has shown that spontaneous and experimental infection with toxoplasma leads to the development of glioma-like tumors(Erichsen & Harboe, 1953; Schuman et al., 1967). Tumors were also detected in 25 out of 1669 free-living birds in the area of Chernobyl and were positively correlated with exposure to radiation(Møller et al., 2013). To assess whether infections, radiation, or even nutritional factors, such as carcinogenic fungal aflatoxins(Imazeki et al., 1995; Uchida et al., 1988) and carnivorous diets(Kapsetaki et al., 2021), are associated with the malignancies and neoplasms of birds in our dataset, a systematic analysis of the carcinogens that these birds may be exposed to in managed settings would be necessary. This would also inform us about potential mechanisms that protect birds from radiation-induced DNA damage(Galván et al., 2014), as well as associations between unpredictable environments and fast life history strategies (e.g. production of more offspring)(Ellis et al., 2009) that explain cancer susceptibility across species.

Conclusions

We explored cancer prevalence across 108 managed species of birds. We found that among the examined life history factors, only clutch size was correlated (positively) with malignancy prevalence and neoplasia prevalence. Our findings are consistent with previous work which looked across 37 species of mammals in managed environments, finding that species with larger litter sizes were more vulnerable to cancer(Boddy et al., 2020a). Further work is necessary, however,to examine whether these patterns hold up in wild and free-ranging populations.

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Author Contributions

This work was part of J.D.'s Barrett's Honors College thesis at Arizona State University. A.A., A.M.B., Z.C., E.G.D., T.M.H., V.K.H., and S.M.R. helped in the collection of neoplasia prevalence, malignancy prevalence, and life-history trait data across species. Z.C. initially analyzed the sex bias cancer data and created the sex bias regression figures. S.E.K. contributed to a later version of the manuscript, reanalyzed data, updated figures, and rewrote sections of the manuscript for final submission to the journal. S.A. contributed in making the age density analyses. A.M.B., A.A., and C.C.M. provided helpful discussions, comments, and guidance throughout the project. All authors commented on the final versions of the manuscript.

CHAPTER 6

NO PETO! BODY SIZE PREDICTS CANCER MORTALITY AMONG PUREBRED DOGS

Introduction: More Than Best Friends

Throughout my dissertation work the only consistent point of disagreement between Dr. Maley and myself was what was to be considered an appropriate title for a forthcoming manuscript. That said, I hope the reader can appreciate the irony that the title of this paper, although suggested by Marc Tollis, came with no objections. *Canis lupus familiaris* is a fascinating case for understanding the tension between natural and *unnatural*, or artificial, selection. Even when compared to plant species, dogs hold the record for the species with the longest history of domestication. Domestication of our soon to be best friends extends tens of thousands of years to the paleolithic period and has resulted in an extreme diversity of phenotypic outcomes(Galibert et al., 2011). Our close accompaniment with dogs, typically for the duration of their life, and our ever increasing vested interest in their healthcare have dramatically increased the study of their commonly occurring ailments.

Another striking commonality that dogs share with humans is their high prevalence of both benign and malignant tumors(Schiffman & Breen, 2015). This circumstance allows for a second recognition to be bestowed, dogs are often cited as the landmark species for comparative oncology studies. Lifetime healthcare surveillance, high propensity for spontaneous tumor generation, and cancer genomic profiles that are highly similar to human malignancies all make dogs the ideal species for comparison. However, to me, is their “natural” evolutionary history

and their subsequent and often divergent domestication pressures that shapes the most compelling story. The major finding in the paper can nearly be entirely extracted from its title, body size is an excellent predictor of the differences in cancer mortality across dog breeds. Yet it is the subfinding within this analysis that more perfectly constructs the evolutionary story I want to tell.

Genomic surveys have proven that all extant dog breeds share a common ancestor dating back some 15,000 years ago(Parker, 2012). It takes no imaginative stretch to say this common ancestor was phenotypically recognizable as what we know today are wolves. Similarly non-imaginative, the first selective steps in their domestication would not have been for the largest phenotypes among this population of now captive wolves. Now, we know for sure that the largest of modern dog breeds, the great dane, was only first bred *at most* 400 years ago. Within an eyeblink of evolutionary time there have been incredibly consequential human-directed modifications to average body size across dog breeds. Among these consequences the most compelling highlighted by the following manuscript is the dramatic spike in sarcoma mortality in larger dog breeds.

There is some variation in time to sexual maturity (also considered the time the animal reaches its adult body mass) across dog breeds but given that all dogs are the same *species* the differences are marginal. A Chihuahua will reach sexual maturity in 6-9 months while a Great Dane will reach it within 18-24 months. Even if we are only considering the connective tissue involved in these two radically different body architectures, the difference in sheer volume that needs to be developed is considerable. I would hypothesize that the body

development of the Great Dane would require some lessening of the controls of cell proliferation within the connective tissues during its developmental period, a lessening that very well could foster a system of cellular apoptotic escape.

Many of our cross-species comparative oncology studies have included some functional cell studies to measure apoptotic sensitivity to induced DNA damage sensitivity. An adjacent experiment that may be useful in validating my above hypothesis would be to culture some osteogenic cells from two dog breeds with significantly different adult body sizes and compare 1) total cell turnover, 2) mutational burden after time period, 3) apoptotic response. Much of osteogenic cell culture requires some 2 and 3 dimensional scaffolding which may make a system like organoids a viable candidate(Akiva et al., 2021; S. Chen et al., 2022).

Abstract

Domestic dogs have been bred for many traits. In some cases, artificial selection for desirable traits has resulted in an increased frequency of diseases such as cancer. The large variation in phenotypic traits across breeds, and the relatively high levels of inbreeding within breeds, provides a natural experiment for exploring drivers of cancer risk within a species. We conducted a meta-analysis of four studies to examine factors associated with cancer mortality across 201 dog breeds, and 181,413 individual dogs, from the UK, Denmark and the US. We found that breed body size explains more variation in cancer mortality than either lifespan or inbreeding. Thus, there is no Peto's Paradox for purebred dogs. Relatively high mortality due to sarcomas in large breeds supports an

evolutionary mismatch hypothesis, in which selective breeding for rapid growth and large body size may have left many modern dog breeds without proper cancer suppression mechanisms such as efficient DNA repair or apoptotic responses.

Introduction

Dogs are humankind's most widespread animal companions and were the first animals to be domesticated before the advent of agriculture (Frantz et al., 2016). Over the course of this mutual history, humans have bred and utilized dogs for working, hunting, and companionship. Selective breeding for highly desirable physical and behavioral traits has resulted in hundreds of modern dog breeds, with 202 breeds recognized by the American Kennel Club and over 300 recognized by the Kennel Club of the United Kingdom. One trait common to many dog breeds is a lifetime risk of cancer that is similar to humans (Schiffman & Breen, 2015), making neoplasia a leading cause of mortality in dogs (Fleming et al., 2011). Certain types of cancers are so prevalent in specific breeds that veterinarians often diagnose the cancer without invasive scanning (Schiffman & Breen, 2015). For instance, brachycephalic breeds such as Boston Terriers, French Bulldogs, and Boxers are particularly susceptible to glial neoplasms and are frequently diagnosed with brain tumors (Song et al., 2013). As the onset and progression of many canine cancers resemble those of human cancers, dogs have been recognized as potentially better models for understanding cancer development (Schiffman & Breen, 2015) than other more widely studied species

such as rodents.

The wide variety of recognized dog breeds vary extensively in their body sizes, which may have important ramifications for their relative cancer risk, and understanding the relationship between breed size and cancer risk may improve preventative and therapeutic measures for cancer in dogs. Theoretically, the greater number of cells in larger and longer-lived multicellular animals should carry a greater lifetime risk of oncogenic mutation (Peto et al., 1975). However, cancer prevalence does not scale with body size across species (an observation dubbed “Peto’s Paradox”) (Abegglen et al., 2015; Boddy et al., 2020b). At the same time, lifetime risk is associated with body size for nonsmoking related cancers (Albanes, 1998) as well as melanoma (Lahmann et al., 2016)in humans. Therefore, while body size does not predict cancer risk across species, it appears to predict cancer risk within species. This opposition of a statistical trend when analyzed within or between groups (or species) is known as “Simpson’s Paradox” (Simpson, 1951). Because dogs are among the most widespread and beloved animal companions for humans, we set out to determine if breed body size is associated with differences in cancer risk. Inbreeding may also play a role in cancer risk across dog breeds, increasing the prevalence of cancer-prone (deleterious) alleles among dogs. High levels of linkage disequilibrium in the dog genome, stemming from the establishment of small breeding populations, has decreased genetic diversity(Sutter et al., 2004), and an excess of deleterious mutations in the dog genome compared to the wolf genome, are likely due to selection for traits other than reproductive fitness in breeding efforts (Cruz et al.,

2008). Several studies have demonstrated the accumulation of deleterious mutations by identifying specific genes that are linked to diseases across breeds(Karlsson & Lindblad-Toh, 2008). To date, there have been few studies on the effect of inbreeding on dog health.

Methods

To determine the dynamics of cancer mortality risk in pure bred dogs, we collected cancer mortality and life history trait data of 201 dog breeds from the literature. The UK Kennel Club conducted two health studies based on questionnaires: one in 2004 sent to breeding clubs(*UK KC 2004 Survey*, n.d.), and another in 2014 sent to registered UK Kennel Club owners(*2014 Pedigree Breed Health Survey*, n.d.). We recorded the total number of dogs (living or dead), the total number of mortalities, the number of cancer incidences in living dogs, and the number of cancer-related mortalities from each individual breed survey summary. We also collected published data from the Danish Kennel Club (Proschowsky et al., 2003), including the total number of breed mortalities and the percent of cancer mortalities. The number of dogs in each breed that died from cancer was calculated by multiplying the cancer mortality rate for the breed by the total number of dogs observed for the breed. A fourth study from the U.S. used information from the Veterinary Medical Database (VMBD), which collected data from 27 Veterinary Medicine Teaching Hospitals in North America during 1984 to 2004 (Fleming et al., 2011). The data was presented as the total number of mortalities per breed and the relative frequency of neoplastic mortality. The number of cancer mortalities was extracted from this data. After collecting the

data from the individual studies, the data was combined by adding each of the observed counts of total mortalities together and the counts of cancer mortalities together. Because some studies categorized breeds differently, some breeds were combined into one entry. For example, one study only presented information for all types of dachshunds (Purebred Breed Health Survey, 2004), while others split dachshunds into several groups including wire- or smooth-haired (Pedigree Breed Health Survey, 2014). These groups were combined into one entry for dachshunds. We calculated the percentage of the total cancer incidence (including living and deceased dogs) and of cancer mortality from the combined values of all four studies.

Heterozygosity data for 150 breeds was extracted from mydogdna.com(*MyDogDNA® — Know Your Dog Better*, n.d.). The percentage of heterozygosity for each breed was recorded when applicable. We also collected inbreeding coefficients from(Calboli et al., 2008), a study analyzing the inbreeding of 10 breeds based on the pedigree of registered UK Kennel Club dogs. As the sources represent different methods of estimated genetic variation, the heterozygosity and inbreeding coefficients were compared based on the ten overlapping breeds through a linear regression. We took information on breed specific body size (lbs), weight class (small, medium, or large), average lifespan, and breed group (sporting, toy, etc.) from the American Kennel Club (AKC) and UK Kennel Club websites (https://www.akc.org/https://www.thekennelclub.org.uk) . If a range of values was given, we used the average value. The different types of cancers for 156 different dog breeds were also collected from the VMBD database. We recorded each type of cancer for each breed, then grouped the types of cancer into three categories: sarcoma, carcinoma, and

hematologic. The percentage of each type out of the total number of malignant tumors was calculated along with each breed separated into body mass ranges, consisting of 0 to 20, 21 to 40, 41 to 60, 61 to 80, and 81 to 180 lbs. This data was used to compare the body mass ranges according to which cancers they were most susceptible to.

We tested for the relationship between cancer mortality and life history variables (body mass, lifespan, and heterozygosity) using simple and multiple linear regressions and phylogenetic general least squares (PGLS) regressions. All analyses were conducted in the R environment(Development Core Team, 2011), and we only included data from breeds for which there were ≥100 total mortalities. Phylogenetic regressions account for the non-independence of model residuals due to common ancestry; we used a recent phylogeny for modern dog breeds (Parker et al., 2017)in the R package caper (Orme, n.d.)and obtained the maximum likelihood estimate for lambda which ranges between 0 (no phylogenetic signal in the data) or 1 (variance completely dependent on phylogeny). We selected the best performing model by comparing each model's Akaike information criterion (AIC).

Results

The numbers of individual dogs, total mortalities, and number of breeds collected from each study are provided in Table 1. The breeds with the highest cancer mortality rates included Irish water spaniel (58.3%), flat-coated retriever (53.8%), Bernese mountain dog (47.9%), and golden retriever (45.6%) (Table 2). The breeds with the lowest cancer mortality rates included miniature pinscher (3.4%), chihuahua (7.5%), Pekingese (7.8%), and Pomeranian (8.4%) (Table 3).

Study	# of Dogs	# of Mortalities	# of Breeds
Pedigree Breed Health Survey (2014)	48,685	5,680	190
Purebred Breed Health Survey (2004)	47,429	14,563	142
Fleming et al. (2011)	80,306	72,376	82
Proschowsky et al. (2003)	4,993	2,928	36*
Total: 362,826	181,413	95,547	201**

Table 1: A Summary of the Total Number of Dogs Observed Across Studies. *Some of the breeds in Proschowsky et. al. (2003) are grouped (ex: Sighthounds). **Total number of unique breeds across all four studies.

Breed	% Cancer Mortality	# of Mortalities	# of Cancer Mortalities	Heterozygosity (%)	Weight (lbs)	Lifespan (years)	Group
Irish Water Spaniel	58.3	103	60	31	56.5	12.5	Sporting
Flat-coated retriever	53.8	873	470	29.5	65	9	Sporting
Bernese mountain dog	47.9	629	301	29.4	94.5	7	Working
Golden Retriever	45.6	5515	2514	32	65	11	Sporting
Scottish Terrier	45.2	564	255	23	20	12	Terrier
Boxer	42.3	1464	620	26	65	11	Working
Bullmastiff	42.1	273	115	29.9	115	9	Working
Bouvier Des Flandres	41.8	225	94	33.5	90	11	Herding
Staffordshire bull terrier	41.0	188	77	33.4	31	13	Terrier
Airedale terrier	40.4	490	198	24.3	60	12.5	Terrier

Table 2: The Ten Breeds with the Highest Cancer Rates. Each breed shown had data for more than 100 individuals.

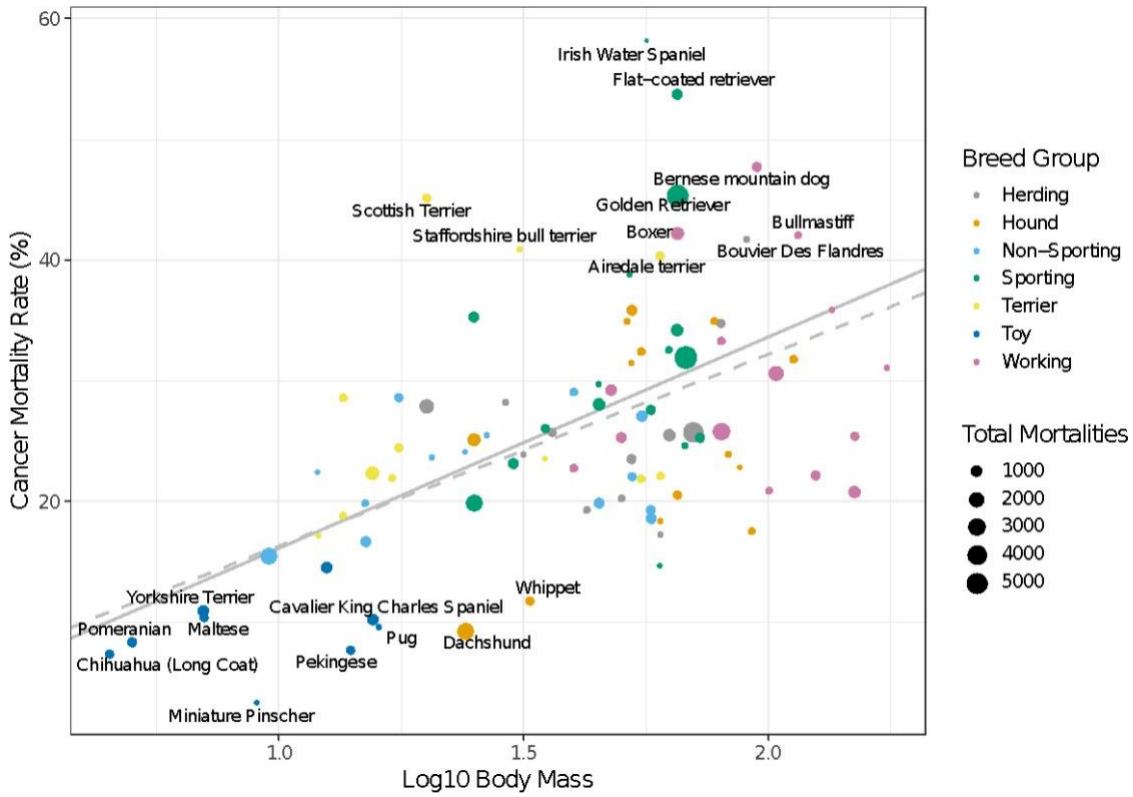

Figure 1: Log_{10} body mass (lbs) as a predictor of cancer mortality rate. Dashed line shows phylogenetic generalized least square (PGLS) line, solid line is uncorrected regression line.

Breed	% Cancer Mortality	# of Mortalities	# Cancer Mortalities	Heterozygosity (%)	Weight (lbs)	Lifespan (years)	Group
Miniature Pinscher	3.4	118	4	34.5	9	14	Toy
Chihuahua	7.5	576	43	39.8	4.5	16	Toy
Pekingese	7.8	552	43	29.5	14	13	Toy
Pomerania n	8.4	617	52	37.3	5	14	Toy
Dachshund	9.4	2574	242	34.7	24	14	Hound
Pug	9.6	187	18	24.6	16	14	Toy
Cavalier King Charles Spaniel	10.3	905	93	25.4	15.5	13.5	Toy
Maltese	10.5	457	48	35.1	7	13.5	Toy
Yorkshire Terrier	11.0	1042	115	36.4	7	13	Toy
Whippet	11.8	541	64	31.3	32.5	13.5	Hound

Table 3: The Ten Breeds with the Lowest Cancer Rates. Each breed shown had data for more than 100 individuals.

Predictors of cancer mortality in dog breeds

Breed body size predicts cancer mortality in dogs, (Figure 1, $R^2 = 0.29$, $p=<0.001$), as does lifespan ($R^2=0.132$, $p=<0.001$); however there is a strong correlation between body size and lifespan in dogs ($R^2=0.3055$). Therefore, when we included both in a multivariate phylogenetic regression, only body size was a significant predictor of cancer mortality (multivariate adjusted $R^2=0.2839$, $p=<0.001$ for body size, $p=0.2363$ for longevity). Heterozygosity is also associated with cancer mortality (Table 4, $R^2=0.05$, $p=0.02$). A multivariate phylogenetic regression of body size, lifespan and heterozygosity reaffirms that, when combined, only body size remains a significant predictor of cancer mortality ($R^2=$, $p=<0.001$ for body size, $p=0.9669$ for longevity, $p=0.2514$ for

heterozygosity.

	Predictor			Overall		
Independent Variable	Predictor	p-predictor value	Coefficients	p-value	R2	lambda
Lifespan	Heterozygosity	<0.01	0.106313	<0.001	0.3246	0.77
	Body Mass	<0.001	-3.1465			
Lifespan	Heterozygosity	<0.01	0.11	<0.01	0.06	0.94
Lifespan	Body Mass	<0.001	-3.15	<0.001	0.31	0.81
Heterozygosity	Body Mass	0.02	-2.78	0.02	0.05	0
Cancer Mortality	Lifespan	<0.001	-0.60969	<0.001	0.2866	0
	Body Mass	<0.001	15.85583			
	Heterozygosity	0.02177	-2.02539			
Cancer Mortality	Lifespan	0.24	-0.17	<0.001	0.28	0
	Body Mass	<0.001	15.25			
Cancer Mortality	Heterozygosity	0.02	-0.61	0.02	0.05	0.46
Cancer Mortality	Body Mass	<0.001	15.86	<0.001	0.29	0
Cancer Mortality	Lifespan	<0.001	-2.03	<0.001	0.13	0
Sarcoma Mortality	Body Mass	0.6297	0.00045	<0.001	0.2913	0
	Lifespan	<0.01	-0.05276			

Table 4: Summary statistics for predictors of cancer mortality.

Types of cancer

The increase in cancers in large dogs is not spread evenly across the

types of cancer (Figure 2). Larger dogs suffer far more sarcomas, the cancers originating in the skeletal and connective tissues. The relationship between increases in body mass and increased sarcoma mortalities in purebred dogs is one of the most striking examples of a single trait explaining much of the variation in risk for a specific cancer type (Pearson's correlation r=0.38, p=5.03e-06).

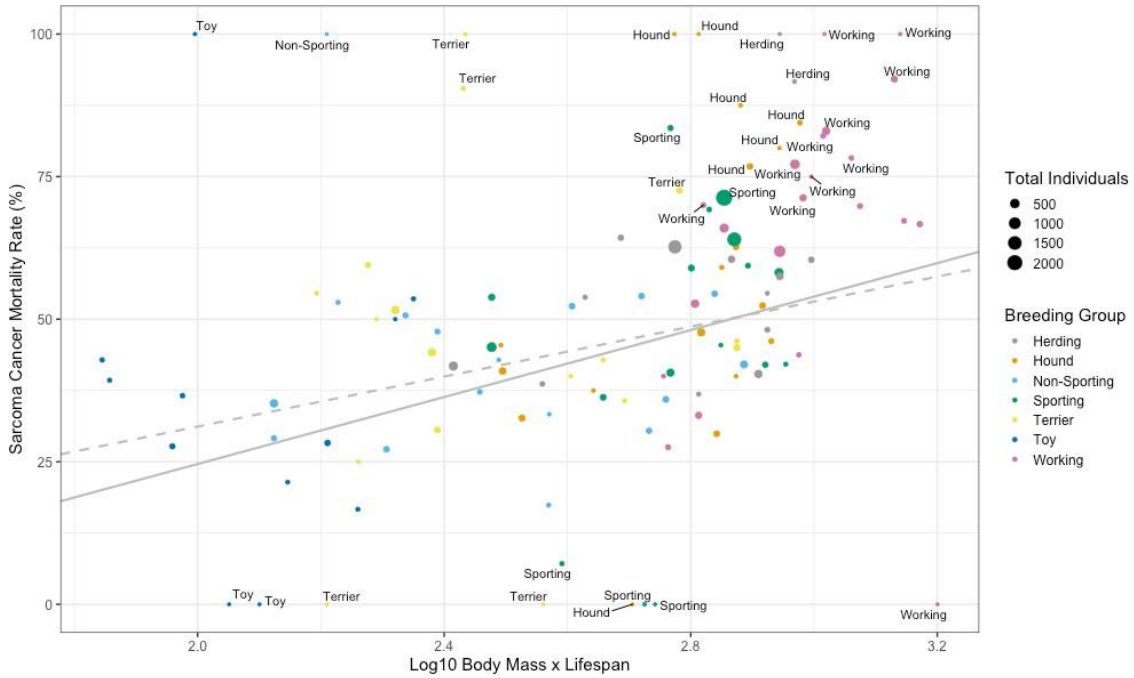

Figure 2: The Log_{10} product of body mass (lbs) and lifespan (yrs) as a predictor of sarcoma cancer mortality. Dashed line shows phylogenetic generalized least square (PGLS) line, solid line is uncorrected regression line.

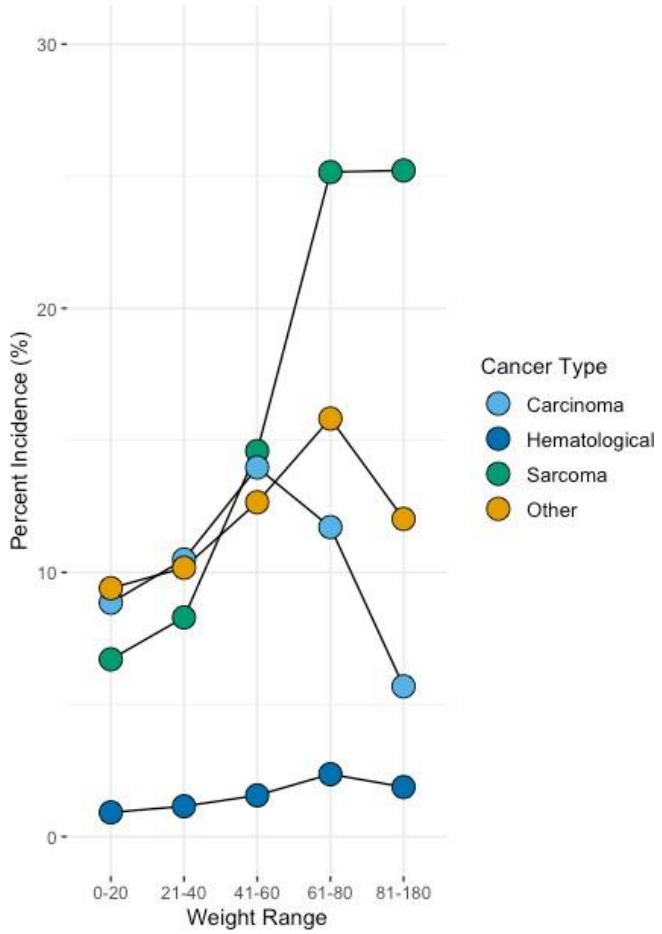

Figure 3: Specific cancer type incidence average weight (lbs) range. “Other” category includes nondescript cancers and glioblastomas.

Discussion

The study of cancer across species is a relatively new field(Aktipis C. Athena et al., 2015), and lineage-specific variation in life history traits may confer different degrees of cancer risk across the tree of life(Boddy et al., 2015). One trait often associated with elevated cancer risk is body size. For instance, height is a risk factor for cancer mortality in humans(Batty et al., 2006; Kabat et al., 2013), with one study showing increased risk of 12% for colorectal cancer, 7% for prostate cancer, and 6% for lung cancer with every 10 cm increase in height(Green et al.,2011). The fact that larger individuals get more cancer may be

due to increased lifetime caloric intake or the expression of more growth factors. Another explanation is that the risk of developing cancer should be a function of the number of cell divisions over an organism's lifetime, and larger individuals are comprised of more cells. However, large and long-lived wild animals do not seem to suffer high mortality rates due to cancer, an observation known as Peto's Paradox. This suggests that these lineages have evolved to somehow suppress cancer and has spawned a new field of comparative oncology(Abegglen et al.,2015; Schiffman & Breen, 2015). However, it appears that there has not yet been enough natural selection on dog breeds to compensate for the higher risk of cancer associated with having a larger body size. Or, in other words, there is an evolutionary mismatch between the large phenotypes we have selected, and canine survival, at least with respect to cancer mortality.

Even the cancer mortality of small dogs (3.4-45.2%) is relatively high for mammals in captivity (ave. 9% from the San Diego zoo)(Boddy et al., 2020b). So,it is not the case that small dogs are protected from cancer, but rather that large dogs are extremely prone to cancer. Studying those large breeds of dogs holds the promise of discovering mechanisms of cancer susceptibility(Khanna et al.,2006), and perhaps even approaches to cancer prevention.

The increase in sarcomas associated with large dogs is reminiscent of the relatively high incidence of sarcomas in human adolescence when the bones are growing the quickest. Large dogs (61-180lbs) reach adult body size at approximately the same age (12-18 months) as small dogs (4.5-20 lbs in 12-15 months). Thus, the bones of large dogs have to grow rapidly. This may require a

relaxation on growth constraints, and perhaps even DNA error checking, that results in an increased risk of bone cancers.

To our knowledge, this study is the first to test an association between inbreeding and cancer mortality. The outlier species in our initial study of cancer rates across species (Tasmanian devils and cheetahs) are famous for having gone through an extreme genetic bottleneck, resulting in very low levels of heterozygosity. This led us to hypothesize that inbreeding might generally lead to an elevated cancer risk. This indeed appears to hold true in dogs, though heterozygosity only explained 0.05177% of the variation in cancer mortality in our meta-analysis. Whether there is an association of heterozygosity and cancer mortality across species remains an open question. The artificial selection that humans have imposed on dogs has resulted in many human desired traits, but has come with the burden of increased cancer incidence, particularly in large dogs. The mechanisms behind this cancer susceptibility remain open questions. Peto's Paradox appears to hold across species(Abegglen et al., 2015), in captivity. So far, in the two cases in which it has been studied, humans and dogs, Peto's Paradox does not hold within species. This may be because, due to gene mixing within a species, larger members of the species do not generally evolve distinct cancer suppression mechanisms from smaller members of the same species. Future studies will have to determine if this is a general pattern within species and whether Peto's Paradox holds for cancer incidence across species in the wild.

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APPENDIX A
A NOTE ON PREVIOUSLY PUBLISHED WORKS

APPENDIX A

None of the above works are currently published in a peer-reviewed journal, although all of them besides *No Peto! Body Size Predicts Cancer Mortality in Purebred Dogs* have been submitted to journals. I am listed as First Author, or Co-First Author, on all these works. All contributing authors have agreed to their publication and inclusion in this dissertation.