Running header: Comparative immunology

The Promise of a Pointillist Perspective for Comparative Immunology

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Abstract

Most studies in comparative immunology involve investigations into the detailed mechanisms of the immune system of a non-model organism. Although this approach has been insightful, it has promoted a deep understanding of only a handful of species, thus inhibiting the recognition of broad taxonomic patterns. Here, we call for investigating the immune defenses of numerous species within a pointillist framework, that is, the meticulous, targeted collection of data from dozens species and investigation of broad patterns of organismal, ecological, and evolutionary forces shaping those patterns. Without understanding basic immunological patterns across species, we are limited in our ability to extrapolate and/or translate our findings to other organisms, including humans. We illustrate this point by focusing predominantly on the biological scaling literature with some integrations of the pace of life literature, as these perspectives have been the most developed within this framework. We also highlight how the more traditional approach in comparative immunology works synergistically with a pointillist approach, with each approach feeding back into the other. We conclude that the pointillist approach promises to illuminate comprehensive theories about the immune system and enhance predictions in a wide variety of domains, including host-parasite dynamics and disease ecology.

Keywords: Comparative immunology, scaling, allometry, immune defense, pace of life

Comparative immunology entails studying the immune systems of non-model organisms from an evolutionary perspective, and it has demonstrated that the immune system is ubiquitous and necessary for all animals to survive (1, 2). It promises a unified understanding of the mechanisms underlying immune defenses and how immune systems are integrated with other physiological systems (1, 2). It has also established important organizing paradigms in immunology, including self/non-self discrimination, the danger hypothesis, and classification of innate vs. adaptive and cellular vs. humoral immune defenses, and placed the origins of specific types of defenses on the tree of life (1–4). Yet, for all its promise, most studies focus on a single or a handful of species and immunology has few frameworks for predicting the specific immune defenses of unstudied organisms and the diversity of immune systems across species. We argue that embracing comparative studies of dozens of species will advance the field of comparative immunology by revealing broad interspecific patterns and the underpinning constraints and selective forces for those patterns.

Two forms of comparative immunology

> ***For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied.***
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> ***~Krogh, 1929, J Am Phys, p 5***

> ***[A] general physiology which can describe the essential characteristics of matter in the living state is an ideal to which we may hope that our successors may attain after many generations, and I want to emphasize that the route by which we can strive toward the ideal is by a study of the vital functions in all their aspects throughout the myriads of organisms. […] the general problem of [a physiological system] can be solved only when … organs are studied wherever we find them and in all their essential modifications.***
>
> ***~Krogh, 1929, J Am Phys, p 4-5***

These two quotes by Krogh illustrate two alternative approaches in Comparative Immunology. The first quote conveys the Krogh Principle (5). It describes the idea that there is one or a few ideal model species for every question. It encourages researchers to find the species

that best allows them to study their question and then apply those discoveries to other species. A broad interpretation of the Krogh Principle is the idea that certain animals, other than traditional model organisms and humans, should be studied to inform human physiology and immunology. This theme of shared biological mechanisms, discovered in one species and extrapolated to all, provides the logic behind studying the innate system of invertebrates to illustrate the innate system of vertebrates, for example (1). This idea has served as a guiding principle for comparative physiology and immunology studies. Indeed, immunological studies are rooted in a comparative approach, from the discovery of phagocytosis in starfish by Metchnikoff (6) to self-recognition mechanisms through allograph and xenograft transplantation in annelids (7, 8). Although the single-species approach has been highly productive, it limits our capacity to make predictions about the immune defenses of other unstudied species that may exhibit unexpected properties not yet observed.

The second quote appears in the same treatise on physiology by Krogh. It reminds readers that a general understanding of any physiological system, including the immune system, requires studying the same functional system in all species with all modifications in all their vivid splendor. It reminds scientists that variation matters as much as the mean and that patterns are missed when one does not investigate immune systems in a diversity of animals in a systematic way. A fully systematic approach requires many points to see the patterns because variation is information. In other words, this is a pointillism perspective of comparative immunology (Fig. 1). Pointillism painting is the practice of "applying small strokes or dots of color to a surface so that from a distance they visually blend together" (9). As an analogy, pointillism illustrates the second of the Krogh quote. The points of color meticulously applied to a canvas are like the targeted data points about the immune system meticulously collected for numerous species. The picture, or broad pattern of between-species variation of the immune system, only emerges when the dots are viewed from a distance. When applied to evolutionary questions, this pointillist perspective is encapsulated in comparative methods in evolutionary biology, which, since Darwin, have remained one of the most enduring and common approaches in evolutionary biology (10, 11).

Together, these Krogh quotes highlight the importance of an iterative approach, identifying unifying principles across all species and identifying novel physiology based on

variation and phenotypic plasticity within and between species. The Krogh Principle expressed in the first quote is a more commonly applied approach in comparative immunology, with discoveries yet to be uncovered by using the latter pointillism comparative approach arising from the spirit of the second quote.

The value of a pointillist comparative approach

Immunology has few organizing principles beyond self/non-self discrimination (12, 13), the associated danger theory (14, 15), and optimality theories (e.g., 16, 17). Ecoimmunology studies often invoke trade-off and allocation theories (18–20), which are forms of optimality theories, for explaining inter- and intraspecific variation in immune strategies. Insights from these hypotheses, however, are often condition-dependent and hard to generalize across species. One could argue that understanding disease and disorders, as is commonly the goal of medical immunology, requires understanding the underlying variation based on how these systems shift under ecological and evolutionary pressure.

Consider, for example, the literature on how nutritional condition shapes immune defenses. Some studies of food restricted individuals demonstrate tradeoffs between immune defenses and other physiological processes such as reproduction (e.g., 18, 19), and studies of individuals with surgically removed fat generally have reduced immune defenses (e.g., 21). Other studies failed to find a reduction of immune defenses in food restricted individuals (e.g., 22). Similarly, results from studies investigating the relationship between individual variation in nutritional condition and constitutive immune defenses within a constraints framework failed to find a consensus, with positive (e.g., 23), negative (e.g., 24), complex (25), or no direct relationships (e.g., 26, 27) depending on the study. This inconsistency among results suggests that a broad pointillism perspective might help identify missing explanatory variables and a broader pattern in the data. Indeed, a meta-analysis investigating the relationship between host condition and infection, which is physiologically regulated by the immune system, found substantial heterogeneity in the strength and direction of condition–infection relationships (28), demonstrating the importance of a pointillist perspective for identifying patterns necessary for interpreting single-species studies. If we gain insight into what conditions are associated with or for which taxa there is a relationship between body condition and immune function, then we can accurately predict when and in which species we will see an association. A goal of the pointillist

approach is to extrapolate from broad immunological patterns to make predictions about immune function in species not as deeply studied as classically evaluated model and non-model organisms studied via a Krogh's Principle perspective.

Transitioning from model organisms to Krogh's perspective to Pointillism

Much time and money have been spent investing in a deep understanding of a few model organisms. Researchers in the field of medical immunology are increasingly aware of the importance of a Krogh's perspective of comparative immunology to advance our understanding of immunobiology, including, but not limited to, the development of the immune system, immune tolerance, infectious disease response, and the evolution of different immune cell classes and proportions (29, 30). However, these studies often rely on an extrapolative approach with significant research invested in a handful of organisms. The number of organisms investigated has increased from using inbred mouse strains to include investigating naked mole rats (*Heterocephalus glaber*), spiny mice (*Acomys cahirinus*), and prairie voles (*Microtus ochrogaster*) for mammals, Burmese python (*Python bivittatus*) and green anole lizards (*Anolis carolinensis*) for Reptilia, zebra finches (*Taeniopygia castanotis*) for Aves, and wood frogs (*Lithobates sylvaticus*) and *Xenopus laevis* for the Amphibia (31–36). These single-species studies still exemplify the philosophy underpinning Kough's Principle. After the great successes of this approach, questions remain about the applicability for generalizing from this relatively small number of species to all species.

In medical immunology, relatively little attention has been paid to evaluating global variation and uncovering the comparative pattern between immune defenses and traits of interest across many species, and more species need to be sampled to extrapolate to the broader pointillist perspective. If a trait shows no variation with another trait of interest, then there is no concern for extrapolation across species based on data collected for a handful of organisms because those relationships are not context dependent. However, we need to understand how variation is patterned to understand the relative importance of pointillist perspective for a particular relationship or endpoint. Still, the question of how best to sample across the tree of life arises with any study involving numerous species to reduce the impact of sampling bias. This sampling concern is analogous to the problem of sampling sub-populations of cells in the brain to understand the complex, 3-D structure of the brain (37) or from any small sample of a

population to the whole. When identifying pointillist patterns in immune defenses, we are not extrapolating cell counts to understand the whole population of cells (37), but extrapolating a predicted immunological response from patterns observed for a sub-sampled number of species. Although evaluating every species is impossible, scientists should assess variation from pilot studies to identify the best sampling approaches and prioritizing studies for targeted, multi-species evaluations.

To begin this ambitious goal and to translate research from model organisms to humans and non-model organisms, more research is needed to fill the gaps in translation from Rodentia to Primates, with perhaps increased efforts in a handful of species, including *Xenopus*, earthworms, birds, and non-model mammals, for evolutionary comparison and to reveal broad patterns. Even so, without evaluating the underlying variation and its distribution, these derived general principles may be inaccurate because more variation among species likely indicates different selective pressure and unique adaptive responses in various species, and more sampling is needed to predict the general principles. Currently, we do not have sufficient species diversity to understand the breadth of the variation in immunobiology, limiting our ability to extrapolate to a new species or even perform power analyses. A pointillist approach can help uncover the underlying pattern of how immune traits change across phylogenetic groups, which is necessary to advance the promise of evolutionary immunology to inform medical immunology (38). Mapping this variation will help us understand when mathematical relationships across species between immune defenses and a trait of interest shift from simple linear relationships to nonlinear relationships, for which is essential for extrapolation.

Scaling of immune defenses as a case study of the pointillist comparative approach

Biological scaling, the study of how traits change with body size (39), provides a promising framework for asking questions about immune systems from a pointillist viewpoint (40–42). Body size likely shapes immune defenses because organismal traits thought to regulate immune defenses (e.g., metabolic rates, 43) and ecological and evolutionary traits that alter parasite exposure and the underpinning cost-benefit of, and thus selective forces on, immune defenses (e.g., sociality, diet, life history, e.g., 44, 45) scale with body mass (46–48). Additionally, if we think of hosts as islands (49), island biogeography theory predicts and data support that large-bodied hosts should have a greater abundance (50), diversity (51–53), prevalence (54), and

absolute biomass (55) of parasites and pathogens than small-bodied hosts (56). However, host islands also evolve, and these differences in parasite pressures in large and small hosts islands should act as a selective pressure to shape the immune system (56, 57) because of the strong selective pressure of parasites on immune defenses (18, 58).

A traditional scaling approach exemplifies the pointillist approach and allows for the application of physical parameters to make predictions about the immune systems of animals of different body sizes. Predicting how the physical structure of an organism and the underpinning physics constrains and shapes immune defenses has the potential to lead to the development of a theory to predict variation of immune defenses across species (40–42, 44). In addition to constraints internal to the organism, biological scaling theory has more recently incorporated constraints imposed by ecological and evolutionary factors through the lens of body size (57, 59–63). Over a century of scaling studies and associated theory (46–48, 61, 64, 65) focused on organismal and eco-evolutionary important traits serves as evidence of the success of such an approach.

Generally, body size scaling relationships are modeled as power laws, $Y = a\,M^b$ (Fig 1A), with linear transformations, $\log_{10} Y = \log_{10} a + b \log_{10} M$ (Fig 1B), where Y is the focal trait, M is body mass and the measure of body size, a is the intercept, and b is the scaling exponent (power law) or scaling coefficient (linear transformation) (48). In the linear transformed model, b describes the slope of the scaling relationship. Many immune defenses are concentration-based, and geometry predicts that concentrations scale as $b = 0$ under isometry. Many traits, however, scale allometrically, such that large organisms have disproportionately greater (hypermetric, $b > 0$) or lesser (hypometric, $b < 0$) concentrations than expected by geometry (Fig 2). **Hypermetric and hypometric curves are nonlinear on their original scale, meaning that if a linear relationship is assumed, predicting the immune defenses of a new species, including humans, from a single species will lead to incorrect extrapolation.** Studies of scaling relationships of immune defenses demonstrate that the architecture of immune systems changes systematically with body mass (56, 66–70), but the type of relationship (isometric vs. allometric) depends on the immune defense in question.

Isometry allows an extrapolation from a single species to others. Perhaps the most intuitive expectation for how concentration-based immune defenses scale with body mass is a prediction

of isometry ($b = 0$), because a reasonable prediction, as is formalized in the Protecton Hypothesis, is that each gram of tissue needs the same amount of protection regardless of the total mass of an organism (71). The Immune Complexity Hypothesis makes the same prediction, but derives its prediction from the delivery of immune components by the fractal architecture of the circulatory system and the rate an immune cell can search a unit of tissue (72, 73).

Some components of the immune system scale proportionally with body mass, that is, isometrically. Antioxidants inhibit free radicals that originate from the respiratory burst of phagocytes during an inflammatory response (74), and, as such, antioxidant concentrations are a proxy of the capacity of the organism to deal with immunopathology arising from an immune challenge (75–77). Antioxidants concentrations were proportional across mammals and birds (78). Similarly, constitutive complement-mediated antibacterial capacity (per mL) against three microbes (*Escherichia coli*, *Salmonella enterica*, and *Micrococcus luteus*) was proportional across >160 mammal species (56), as were lymphocyte concentrations in birds (67) and reptiles (70). Lymphocyte concentrations in mammals had a slope slightly less than but very close to 0 (b [95% credibility interval]= 0.04 [0.08 to 0.02]; (79)). Because blood volume scales isometrically with body mass in mammals (80, 81), mammals of different sizes have close to proportional total numbers of lymphocytes. As constitutive concentrations of these immune defenses are regulated tightly by complex signaling and feedback cycles (82–84), these data imply that the regulatory set-point for these defenses did not change with body size despite the larger absolute surface area of large animals that is expected to provide a greater absolute invasion surface for parasites. The remarkable proportionality of these defenses with body mass is a pattern that could not be expounded upon without the pointillist comparative perspective, but it also means that studying the dynamics of these systems in one species may be more easily translated to understanding the dynamics in another species, including humans, because the responses are linear. Further, translation and application would also be more accurate in datasets with narrow variation around the regression line.

A caveat to these conclusions is that lymphocyte concentrations and antibacterial capacity constitute parts of the more extensive, complex, multifaceted immune system rather than the overall architecture of the integrated network, and the nuances may matter. For example, lymphocytes are identified and counted by morphology and consist of functionally different cell types (e.g., B cells, helper T cells, cytotoxic T cells, regulatory T cells, natural killer cells) that

cannot be distinguished by simple morphology-based leukocyte differentials (85). If the relative proportions of these different types of lymphocytes scale differently, then an extrapolation from a single species, such as a lab mouse, to another species becomes more challenging without understanding the broader pattern of scaling (66).

A pointillist perspective is necessary to predict immune defenses that scale allometrically. Allometric scaling describes patterns of traits that change predictability, but disproportionately with body mass, making extrapolation from a single species to another species challenging without first defining the overarching pattern. For example, a recent comparison of circulating heterophil concentrations of 126 reptiles and 115 birds with circulating neutrophil concentrations in 244 mammals revealed that concentrations for these functionally equivalent leukocytes are shaped differently by body mass in these groups (70), although the mechanism by which this occurs is unknow. The slope of the line describing the relationship between body mass and granulocyte concentration (both $\log_{10}$-transformed) was indistinguishable from 0 for reptiles (b = 0.02 [-0.01, 0.05]), steeper for mammals (b = 0.11 [0.09, 0.15]), and even steeper for birds (b = 0.20 [0.16, 0.25]; Fig 3; (70)). Consequently, an assumption of proportional scaling for reptiles would lead to a reasonably correct prediction of the mean granulocyte concentration for any reptile species regardless of the mass of the organism used as a reference concentration or the mass of the predicted organism because of the isometric scaling of granulocyte concentrations. However, applying that same assumption of isometric scaling to granulocytes concentrations to mammals and birds would lead to incorrect estimates if the reference animal were a different mass than the animal estimated (Fig. 3). For example, on average, a 30-g mammal has a neutrophil concentration of 2.2×10^9 cells / L. Extrapolating from a 30 g mouse to a 35,000-g spotted hyena (*Crocuta crocuta*) under the assumption of isometry, predicts that the hyena will also have a neutrophil concentration of 2.2×10^9 cells / L. Using the allometric equation derived from the data, however, predicts that the hyena will have a 4.78×10^9 cells / L, which is 2.17-fold greater than the prediction from isometry. The discrepancy would be even greater for heterophils in birds because of the steeper allometric relationship. Extrapolating from a 30-g budgerigar (*Melopsittacus undulatus*) with a heterophil concentration of 2.47×10^9 cells / L leads to a prediction of 2.47×10^9 cells / L for a 25,000-g emu (*Dromaius novaehollandiae*), but the hypermetric relationship derived from the data lead to a prediction of 10.07×10^9 cells / L, a

4.11-fold difference. This example also demonstrates that a lack of body scaling equivalency among taxonomic groups means that using the scaling equation from one group to make predictions about another group will lead to incorrect predictions.

The pointillist perspective allows the development of questions that are not obvious from studies of a single species. For example, the convergence of granulocyte concentrations at 30 g stimulates speculation about the cause of this convergence at that body mass. The antimicrobial activities of granulocytes, including the release of host- and pathogen-damaging oxidative species during degranulation, may cause immunopathology (77, 86, 87). One could speculate that large animals can withstand this damage more than small ones simply by having more cells, a form of passive tolerance (42). This idea is supported by a within-species study of green tree frogs (*Litoria caerulea*), demonstrating that large frogs are more likely to survive *Batrachochytrium dendrobatidis* (*Bd*) infections because their larger size lessens the energetic and ion-loss costs of infections (i.e., damage) simply because of allometric effects of size (88). A similar mechanism may explain the greater *Bd*-associated survival in larger common toads (*Bufo bufo*) (89).

These interspecific patterns also provide evidence for speculating how differences in disease risk and immune systems in these groups drive these differences in scaling relationships among reptiles, birds, and mammals. As the primary bactericidal and fungicidal leukocyte in mammals (90, 91), granulocytes are mandatory for life, form a crucial part of the first line of defense against pathogen invasion through direct antimicrobial activities, and help orchestrate B and T cell responses (91–93). In mammals, neutrophils kill microbes directly by three mechanisms: phagocytosis (87, 92), the release of antimicrobial molecules via degranulation (94), and the expulsion of chromatin nets during NETosis (95, 96). Differences in the architecture of the immune systems among groups provide one of many possible explanations for differences in the scaling of granulocytes among these three groups (67, 70, also see these papers for alternative hypotheses). For example, birds differ from mammals in the antibacterial molecules used in their degranulation response, so their granulocytes might be less potent than mammals (67, 97), and reptiles have other cell types that perform some of the same functions as granulocytes (70, 98). Among other differences, reptiles investigated have a greater proportion of phagocytic B1 cells than mammals and birds (70, 99–101), and they have azurophils that can

resemble monocytes or neutrophils and are lacking in birds and mammals (70, 102). These results indicate that reptiles and terrestrial endotherms have solved the problem of defending different-sized bodies from pathogens differently (70), an insight impossible to make without analyses rooted in a pointillist comparative approach. The results also inform drug development and predictions of dynamics of newly emerging diseases in communities and spillover risk to humans.

A pointillist perspective allows the testing of theory. The hypermetric scaling of granulocyte concentrations in mammals (66) and birds (67) is consistent with the Safety Factor Hypothesis, a data-driven hypothesis that posits that large organisms need greater immune defenses than small ones specifically because their largeness disproportionately increases the risk of exposure to disease-causing agents and disproportionately decreases their relative rates of cell replication necessary for many induced immune response relative to the replication rate of a small parasite, pathogen, or virus (66). Simultaneously, the costs of the failure of the immune system for a large organism—that is, the failure of the immune system to clear or contain a challenge—is disproportionately greater relative to costs for a small organisms because it takes more time to become large and to reproduce (66, 103). Therefore, large organisms should invest in safety by having disproportionately greater immune defenses, and size should shape the architecture of the immune system (66). The Safety Factory Hypothesis specifically predicts body-size mediated variation in immune defenses that is not proportional, requiring a Pointillist perspective.

Perhaps the most striking thing about the scaling of all the immune defenses measured is that few relationships are consistent with the predictions derived for metabolic rates alone. The Metabolic Rate Hypothesis (104) is derived from the Metabolic Theory of Ecology (105), and it posits that basal metabolic rate (BMR) constrains the rates of cellular processes (104). By extension, concentration-based immune defenses, including cell concentrations and antibacterial defenses, should scale with body mass with the same slope as mass-specific BMR, which is most often reported as -0.25 (106), and whole-animal cell counts should scale with whole-animal metabolic rate, often reported at 0.75 (105). Although hematopoietic stem cell pools across mammals (104) scales as expected based on metabolic rates ($b = 0.75$ for total cell count and whole-animal metabolic rate), most investigated immune defenses are concentration-based and scale isometrically ($b \sim 0$) or hypermetrically ($b > 0$) (see references in 42, 66). In addition to

hypermetric and isometric scaling of bird (67), reptile (70), and mammal (66) leucocytes described previously in this section, innate and adaptive responses to West Nile virus scale with a slope of ~0 (40, 107, 108), as does plasma-mediated killing of microbes in birds (109, 110). Isometric and hypermetric slopes are steeper than the slope predicted by the Metabolic Rate Hypothesis (-0.25). Even immune defenses with hypometric scaling ($b < 0$), as seen in the scaling of mammal lymphocytes (b = -0.04) (66), are substantially steeper than the predicted -0.25.

The scaling coefficient for metabolic rates is not always -0.25 and depends on the range of body masses examined (111–113) and the groups examined (60, 114). When the observed pattern for the scaling of metabolic rate is not -0.25, the logic of the Rate of Metabolism hypothesis leads to the prediction that the scaling of immune defenses should match the observed pattern of scaling for metabolic rates. It follows that the discrepancy between the scaling coefficient of immune defense and that for metabolic rate is even greater if the coefficient for mass-specific BMR is the often documented -0.33 (61, 115, 116) instead of -0.25. These observations, arising from a pointillist perspective, leave open the question: how are large terrestrial vertebrates maintaining concentrations of immune defenses equal to or higher than those maintained by small counterparts given the expected constraints of metabolic rates (56)? As most immune defenses studied to date are constitutive, this pattern could be explained by the commutative accumulation of cells or antimicrobial molecules in circulation over time (56, 70). That is, the cell concentrations and antibacterial activity measured may reflect the equilibrium concentration of these immune defenses, which are regulated by negative feedback cycles (56, 83, 117). Comparative data across numerous species on rates of proliferation during induced immune defenses and regulatory networks are necessary to resolve these outstanding questions.

Pointillism leads to new, testable questions not revealed using a Krogh's Perspective. A consequence of the different scaling patterns of lymphocytes and granulocytes, heterophils in birds and neutrophils in mammals, is that the species shift from lymphocyte to granulocyte dominant as body mass increases. We analyzed the scaling of species means of neutrophil to lymphocyte ratio in mammals (n=259) and heterophil to lymphocyte ratio in birds (n=116) (Data from 67). We applied the modeling approach of Ruhs and colleagues (67) using Bayesian phylogenetic mixed effects linear models to compare the scaling relationship of two groups

within a model selection framework. These models demonstrated that the intercept and scaling coefficient for birds and mammals are not appreciably different (Fig. 4), and that granulocyte to lymphocyte ratios in both groups share an intercept of -0.42 [95% credible interval: -0.78, -0.09] and a scaling coefficient of 0.14 [0.11, 0.17]. Phylogeny explained 71.4% [55.3, 83.4%] and body mass explained 14.4% (8.0, 23.4%) of the variation. Although 14.4% appears small, it represents 50% of the variation after accounting for phylogeny. The model indicates that body mass shapes the relative circulating concentrations of these leukocytes similarly in birds and mammals, and, on average, birds and mammals switch from a lymphocyte-dominated to a granulocyte-dominated strategy at 1000 g.

Within the context of switching from a lymphocyte-dominant system to a heterophil-dominant system, a pointillist perspective allows one to ask if there is a functional consequence of a shift to a granulocyte-dominant phenotype. Do species with a granulocyte-dominated phenotype have an immune system more prone to experiencing inflammatory responses and do they experience more autoimmunological pathologies? Given the central role of granulocytes in direct antimicrobial activities, priming adaptive immune responses, and diseases, and the tight regulation of neutrophil concentration in the circulating (migratory) pool (92, 93, 118), it seems unlikely that these size-based shifts do not have functional consequences for the organism.

The pointillist approach allows for choosing better models for humans. Choosing appropriate model organisms to represent humans is critical for translating findings to interventions for human health, a primary goal of many medical immunology studies. Let us use granulocyte to lymphocyte ratios as an example. Given the role neutrophils play in cancer (119), inflammation (120, 121), sepsis (122, 123), autoimmune diseases (124–126), and disease and health in general (127), nonlinear scaling of neutrophils in mammals likely has consequences for picking accurate models for understanding human diseases and patterns of cancer across species.

First, from a Krogh's Principle perspective, mice, the most typical animal model in medical immunology studies, have a lymphocyte-dominated granulocyte to lymphocyte ratio (C57BL/6L: male: 0.50; female: 0.36; calculated from 128), whereas humans have a granulocyte-dominated granulocyte to lymphocyte of 2.22 (Calculated from 129). Given this discrepancy, a Krogh's Principle perspective indicates that we might require caveats for extrapolating from mouse immune dynamics to those in humans. A comparison of a single

407 species with humans, however, leaves open the question of what species would serve as a better
408 model for humans and whether humans are just outliers to patterns across species. A pointillist
409 perspective provides a framework for answering these questions.
410 As a starting point, a pointillist perspective allows us to ask if humans have the
411 granulocyte to lymphocyte concentration expected for their body size. Humans were not
412 included in the scaling analysis of granulocyte to lymphocyte concentrations, allowing us to use
413 the model to predict the granulocyte to lymphocyte concentration ratio for humans. Plugging the
414 average mass of a human (66.5 kg, 130) into the equation from the scaling model predicts a
415 granulocyte to lymphocyte ratio of 1.80 for an average human. Thus, humans have 1.23-fold
416 more circulating granulocytes relative to lymphocytes than expected for their size, although this
417 value is within the 95% CI. Indeed, a human has the granulocyte to lymphocyte ratio of an
418 average 215.8 kg mammal, an animal with a mass that is 3,785-fold greater than a human and
419 that weighs slightly less than an average red deer (*Cervus elaphus*; 241 kg). That humans have a
420 disproportionately greater granulocyte to lymphocyte ratio for their body size, is not evident
421 without a pointillist perspective. A scaling approach suggests that species with a granulocyte to
422 lymphocyte ratio similar to humans (e.g., black-tailed prairie dog, *Cynomys ludovicianus* G:L
423 ratio = 2.57, mass = 797 g) might be a better model for immune dynamics of circulating
424 granulocytes and lymphocytes in humans. This exercise exemplifies how a scaling framework
425 can aid in understanding which species have disproportionately greater or lesser immune
426 defenses for their size and in finding better matched single-species models for modeling human
427 immune defenses.
428
429 ***Pointillist tests of the pace of life hypothesis and ecological influences on immune defenses***
430 Scaling is not the only possible pointillist approach in comparative immunology. When
431 variation in immune defenses exbibit an allometric relationship, questions arise as to what
432 selective pressures or trade-offs may exist that create different adaptive balances in
433 multidimensional spaces (60, 61). One example of research being developed in this framework is
434 based on the pace of life hypothesis, which posits that species with slow-paced life histories
435 (long-lived species with low annual reproduction) should invest in immune defenses differently
436 than those with fast-paced life histories (short-lived species with high annual reproduction) (43,
437 44). It posits that slow-paced species should evolve immune defenses that are more specific and

less inflammatory and that prioritize induced rather than constitutive defenses because this strategy minimizes autoimmune costs and maximizes longevity (44). Pointillist comparative approaches are necessary to test whether broad patterns support the hypothesis. Consistent with this hypothesis, pointillist comparative studies have demonstrated that heterophil to lymphocyte ratios become less dominated by inflammatory heterophils with increasing life span in 239 species of birds (131). Similarly, natural antibodies were positively associated with incubation period (n = 70 species, 110), and bacteria-killing ability was negatively associated with mass-corrected BMR in tropical birds (n = 12 species, 109). However, a study of 6 species of *Peromyscus* mice found that immune defenses were not constrained to a single dominant axis and not determined solely by the pace of life (132).

A pointillist approach also allows for testing competing hypotheses. Data collected from dozens of species allow for the testing of the relative importance of different covariates in explaining variation in immune defenses allowing the testing of different hypotheses. For example, a measure of the adaptive immune system (MHC gene copy number) is negatively associated with a measure of the innate immune system (complement-mediated hemagglutination) across 37 species of birds, implying an evolutionary trade-off between the innate and adaptive immune system (133). Although MHC gene copy number was negatively associated with body mass as predicted by the pace of life hypothesis, the number of MHC gene copies was not associated with lifespan, breeding latitude, or extent of migration (133), suggesting that body size rather than life history and ecology might be mediating these immune defenses across evolutionary time. This idea is further supported by studies that directly compared the predictions of the pace of life hypothesis with those from scaling hypotheses. Life history (maximal longevity and maximal reproductive potential in mammals, birds, and reptiles) and ecological traits (sociality and trophic level, mammals only) did not explain interspecific variation in granulocyte and lymphocyte concentrations better than body mass alone, and their addition to models with body mass increased the explanatory power of models minimally (mammals, birds), if at all (reptiles) (66, 67, 70).

In contrast, a study of 24 species of neotropical bats demonstrated that leucocytes per visual field increased with body mass and were greatest in carnivorous bats and that bacteria-killing ability decreased with increasing roost permanence and protection (134). Pointillist

studies of mammals also demonstrate that the scaling of proportions of granulocytes circulating in bats differ from the proportions in non-volant mammals and birds, suggesting that the unique immune defenses of bats are not driven by flight alone (68). Together, these pointillist studies support the idea that bats have novel immune systems, affecting their ability to act as a reservoir for zoonotic diseases (135). They also speak to the ability to test numerous eco-evolutionary frameworks for interspecific variation in immune defenses and the need to collect data from numerous species to test those frameworks and understand the relationships among them. These cases also provide examples for how incorporating variables other than body mass into scaling models allows for disentangling the effects of body mass from other effects.

Synergy between the two comparative approaches.

> ***"In the absence of comparative studies, an entire field may be led astray by observations that are either species specific or misinterpreted in the absence of comparative data"***
>
> ***Yartsev 2017 p467***

> ***"…the comparative approach serves as an extremely powerful tool to assess the validity of universal principles on a case-by-case basis."***
>
> ***Yartsev 2017 p468***
>
> (Juxtaposition of these quotes is from reference 136)

Krogh's Principle and the Pointillist Approach can synergistically inform each other (Fig. 5), and both are necessary to understand how the genome and environment translate into immune phenotypes and their consequences for human and animal health and disease dynamics. The pointillist perspective can shape questions that are best addressed by single-species studies conducted within the spirit of Krogh's Principle. Many of these ideas were elucidated above, but briefly:

- Patterns identified from a pointillist perspective can be used to make predictions about species not yet studied, which can then be tested by measuring the new species as demonstrated by the analysis of the human granulocyte to lymphocyte ratio herein.

- Species with large residuals identified by the pointillist comparative approach can be investigated within a Krogh's Principle approach. For example, the species with high residuals of the scaling relationship for G:L ratio can be investigated as models for sepsis (inflammatory responses in the blood).
- Pointillist approaches can be used to ask how traits change with other potential explanatory variables after accounting for phylogeny (137–139), as demonstrated for the scaling of granulocyte to lymphocyte ratios for birds and mammals herein. After accounting for phylogeny, body mass explained ~50% of the remaining variation in the ratio of these leukocyte concentrations. Similarly, after accounting for phylogeny, Minias et al. (133) found a negative correlation between indicators of innate and adaptive immune defenses. These patterns can be further explored within a single species to understand mechanisms underpinning the pointillist patterns.
- Ecological and evolutionary hypotheses about immune defenses often arise from studies of variation within or between populations, and they can be tested with more substantial power within a pointillist framework. After accounting for one variable of interest, one can ask, what common ecological and evolutionary traits do species with large negative or positive residuals share? For example, a meta-analysis of the costs of responses to induced immunological challenges found that animals incur costs of immune activation, but small species that are relatively long-lived incur the largest costs, presumably because of how the cost-benefit structure changes with body size and life history traits of animals (140). This example demonstrates the need for a pointillist approach to reveal ecological and evolutionary patterns that are impossible to see within a single species.

Studies of a single or few species within the spirit of Krogh's Principle feedback into pointillist studies:

- Perhaps the most apparent synergy is that data collected during studies of one or a few species provide the data for extensive comparative studies. Studies of scaling of leukocyte concentrations and proportions (66–70) and those of how hemagglutination (133) and leukocyte ratios (131) evolved with life history traits exemplify this idea, as those studies compiled data from single-species studies.

- Deep and detailed knowledge obtained from studies of focal species can inform the design of pointillist studies, ensuring data are comparable across species. For example, immune assays are often calibrated for a specific species (56), as exemplified by assays to measure microbial killing ability, a measure of the capacity of serum, plasma, or whole blood to kill a microbe *ex vivo*, which are often calibrated so that dilution of sample kills, on average, approximately 50% of the microbe used in the challenge (141). Thus, widely different parameters are used in single-species studies. For example, approximately 50% of 20 µl of an *E. coli* solution (~5,000 bacteria ml^{-1}) was killed by Dall sheep (*Ovis dalli dalli*) sera samples diluted 1:2 in Luria Bertani (LB) broth (142), but sera from North American elk (*Cervus elaphus*) could be much less concentrated,1:40 in LB, and still kill the same amount of bacteria (26). However, a comparison across species requires the implementation of dilution curves to capture the variation among species. For example, Heinrich and colleagues (143) used an 8-dilution curve to compare antimicrobial activity in samples from 10 species. Similarly, Claunch and colleagues (144) proposed a standardized 12-dilution curve to compare antimicrobial activity across species; they demonstrated that waiting longer to freeze a sample diminished antimicrobial activity in blood produced from some birds but not the tested reptile and mammal species. The application of this approach facilitated the previously discussed scaling study of antibacterial capacity against three bacterial species in >160 species of mammals (56).
- Tools developed for studying the immune system in one species can be adapted for studying the immune system in many species, allowing pointillist comparisons. In particular, understanding how genotypes translate to phenotypes through -omics approaches will help illuminate immune defenses and their associated networks (145, 146). The proliferation of annotated genomes (e.g., 147) and -omics tools provide an opportunity to collect the data necessary for pointillist comparative studies of complex immune defenses from genotype to phenotype. For example, Hawash and colleagues demonstrated that apes (n=2 species) mount stronger and less specific early transcriptional responses to both viral and bacterial stimulation than African and Asian monkeys (n=2 species), with implications for understanding the robust innate immune responses of humans to similar challenges (148).

Immunology has gained significantly from comparative approaches. To achieve the maximum potential of the promise of comparative immunology, data from both a deep exploration of selected model organisms and targeted explorations of patterns across hundreds of species will be needed to provide a clearer picture of the evolution and function of our physiology.

Acknowledgements

We thank L. Martin, J. Brinkworth, C. Briggs, J. Drew, J. Harrison, C. Ghalambor, J. Adelman, and B. Wolf for critical conversations around these ideas. We thank two anonymous reviewers for feedback on an earlier version of this manuscript.

Grants

Research/Work reported in this publication was supported by SUNY System Administration under SUNY Research Seed Grant Award RFP #23-01-RSG (to CJD) and National Institute of Environmental Health Sciences 5R01ES034829 (to MES).

Disclosures

The authors have no conflicts of interest to report.

Author Contributions

CJD conceived the thesis for this review, analyzed data, and prepared figures. CJD and MES interpreted the results, drafted and revised the manuscript, and approved the final version of the manuscript.

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Figure captions:

Figure 1. Every point on a pointillist style painting contributes to the emerging image when viewed from a distance, just as data on individual species contribute to an emergent understanding of predictive patterns of interspecific variation of immune defenses when viewed together and from a distance. (Image purchased from iStock.com/GlobalP and modified using elektrobild.org)

Figure 2. Hypermetric (b>0, purple lines), isometric (b=0, black lines), and hypometric (b<0, red lines) scaling patterns for **concentrations** on (A) linear and logarithmic (B) axes. Patterns of allometric (hyper- and hypometric) scaling are non-linear on the original axis, meaning that extrapolating understanding about the immune system from a single species to make predictions about another species is challenging.

Figure 3. Granulocyte concentrations in blood scale isometrically in lizards ($granulocyte\ concentration = 2.29\ (body\ mass)^{0.02}$), but hypermetrically in mammals ($granulocyte\ concentration = 1.51\ (body\ mass)^{0.11}$) and birds ($granulocyte\ concentration = 1.24\ (body\ mass)^{0.20}$). Consequently, and because the taxonomic groups share a similar intercept, 30-g individuals of each group have similar granulocyte concentrations (black vertical line on the inset), but a 35,000-g mammal has a 1.8-fold greater concentration than a 35,000-g reptile and a 35,000-g bird has a 3.5-fold greater concentration than a 35,000-g reptile. An assumption of proportional scaling for lizards would lead to a reasonably correct prediction of the mean granulocyte concentration for any species regardless of the organism's mass used for the initial measurement or the mass of the predicted organism because the concentration of granulocytes scale isometrically. However, applying that same assumption of isometric scaling to granulocytes to mammals and birds would lead to incorrect estimates if the animal used as a template is a different mass than the animal estimated (green asterisk = mammal estimate under isometry; orange asterisk = bird estimate under isometry). Modified from (70).

Figure 4. Granulocyte to lymphocyte ratio increases disproportionately with body mass, and the relationship is indistinguishable for birds and mammals: $log_{10}(granulocyte\ to\ lymphocyte\ ratio) = -0.42\ (-0.78, -0.09) +\ \ 0.14\ (0.11, 0.17) \times log_{10}(body\ mass)$ (A). The simplest model for the scaling of granulocyte to lymphocyte that has a DIC value within 5 points of the lowest DIC value did not have a different intercept or slope for birds and mammals (B).

Modeling was performed following Ruhs et al. (67). Briefly, Bayesian phylogenetic linear mixed-effects models were built using the mcmcGLMM procedure in Program R (149, 150). The response variable was $\log_{10}$-transformed granulocyte to lymphocyte ratio (data from 67). The fixed effects for each model are presented in the table (B). Phylogenetic covariance matrix for this analysis was estimated using a phylogenetic tree constructed with National Center for Biotechnology Information molecular data and phyloT (151), and polytomies were resolved using the randomization process in phyloT. Model chains were run for 7.8×10^5 iterations, a 180,000-iteration burn-in, and a 600-iteration thinning interval. We estimated unadjusted phylogenetic heritability to measure how much of the total observed variation was explained by phylogeny (152) and calculated marginal R^2 as a measure of how much of the total variation was explained by the fixed effects (153). Relative support for each model was determined based on DIC values and model differences.

Figure 5. Synergistic interactions between the two comparative immunology approaches with an example. (Figure created with BioRender; graph of neutrophil data was modified from (66))