Footprints of functional decline: using complementary physiological and behavioural biomarkers as proxies for population dynamics over space and time

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Running headline: Functional decline: an integrated biomarker approach

Key words: macrophysiology, gut health, microbiome, conservation, endocrinology, social networks, thyroid hormone, glucocorticoids

Article Type: Issues and Perspectives

Word count: abstract 198; text 5955
References: 131
Figures: 5
Tables: 1

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Data accessibility: There are no primary data presented in this Issues and Perspectives piece.

Author Contribution Statement: The manuscript was conceived by SS. JB and SS had an equal contribution to manuscript drafts. All authors contributed to refining the framework, editing drafts and preparing figures.

Abstract

Linking environmental conditions to the modulators of individual fitness is necessary to predict long-term population viability and resilience. Behavioural and physiological biomarkers can provide this mechanistic insight into how individuals perceive and respond to environmental challenges through primary physiological responses, secondary downstream responses and tertiary whole organism responses. To fully exploit biomarkers, we need to move beyond single biomarker studies to develop an integrative approach that models the interactions between extrinsic challenges, physiological and behavioural pathways and their modulators. Here we introduce two frameworks for using multiple integrated biomarkers to establish changes in functional condition. The *Functional Marginality Hypotheses* proposes that relative changes in allostatic load, reproductive health and behaviour can evidence and establish causation driving macroecological processes such as local extirpation, colonisation, population dynamics and range change. The *Functional Recovery Hypothesis* proposes that a similar approach can serve as a valuable conservation tool for evaluating individual and population level health, predicting responses to future environmental change and measuring the impact of interventions. We highlight specific studies that have used complementary biomarkers to link extrinsic challenges to population performance. This framework of integrated biomarkers has untapped potential to identify causes of decline, predict future changes and mitigate against future biodiversity loss.

Introduction

One in five vertebrate species are classified by the IUCN as vulnerable, endangered or critically endangered (IUCN, 2013). These declines and losses are largely attributed to anthropocentric changes in the environment such as land conversion, climate change and unsustainable natural resource harvesting and extraction. Population decline and range contraction occur where populations are no longer viable because of emigration or reduced survivorship or reproduction of resident individuals (Gaillard *et al.* 2000). In small populations, intrinsic issues such as inbreeding depression, mate incompatibility, reproductive pathologies and poor condition are important drivers of poor fitness, defined as the relative ability to leave viable offspring. However, the initial drivers of population decline are often extrinsic threats such as habitat loss and fragmentation, pollution, predation, disease, harvesting, or persecution (Brook *et al.* 2008) that lead to reduced fitness.

One key to halting biodiversity loss lies with identifying these mechanistic precursors of extinction, population decline and range contraction (Chown & Gaston 2008) as well as biotic interactions (Trevelline *et al.* 2019) including disease presentation (Hing *et al.* 2016). Individuals can buffer the impact of environmental challenges with behavioural or physiological responses. Allostatic load is the cumulative result of altered and sustained changes in physiology in response to environmental challenges, which when chronic or repeated stressors are energetically too costly for an individual to buffer causes allostatic overload (McEwen & Wingfield 2003). The assumption is that chronic extrinsic challenges cause cascading effects that can lead to reproductive and immune suppression. However, we currently have limited evidence for the links between environmental stressors, physiology, behaviour and fitness measures that can help predict both individual and population level responses to challenges (Cooke *et al.* 2013; Beehner & Bergman 2017).

Given the scope for physiological and behavioural biomarkers to act as windows into how organisms perceive their environment (Wikelski & Cooke 2006), a range of biomarkers have been developed for sampling physiological and behavioural states (Madliger *et al.* 2018). Their relevance to ecological questions has been reviewed in several recent papers together with the most appropriate methods for their use (Cooke & O'Connor 2010; Cooke *et al.* 2013; Sopinka *et al.* 2016; Madliger *et al.* 2018). Although many ecophysiology studies use single biomarkers to assess responses of individuals within populations to environmental challenges, there remains an untapped potential to use suites of complementary biomarkers to assess population level responses to external challenges (Madliger *et al.* 2018). In this paper, we describe biomarkers from a range of biological pathways that provide complementary streams of information about responses to environmental challenges. We then discuss how these tools can provide macrophysiological data to answer ecological questions over large temporal and spatial scales. We also highlight studies that have used multiple biomarkers linking physiology, behaviour and fitness and show how this approach can test predictions stemming from two hypotheses which support theoretical and applied work.

Stress pathways and fitness consequences

Primary 'stress' response

Our understanding of the molecular, biochemical, physiological and behavioural mechanisms that organisms employ to tolerate stressors is built on the pathways that maintain homeostasis (Somero, 2002; Madliger *et al.,* 2018). Biomarkers along these pathways can be metabolic fingerprints of the primary intracellular regulatory signallers or they may be

105 secondary extracellular footprints of down-stream by-products or tertiary responses to the
106 signallers (Dobson and Smith 2000; Dobson *et al.,* 2003; Martin, 2009). A key primary stress
107 response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis in birds and
108 mammals or the hypothalamic-pituitary-interrenal (HPI) axis in fish, amphibians and reptiles,
109 which leads to the release of glucocorticoids (GCs) and catecholamines (Möstl and Palme,
110 2002; Sopinka *et al.*, 2016; Beehner and Bergman, 2017; Palme, 2019). GCs are steroid
111 hormones that up-regulate glucose production and suppress immune and inflammatory
112 responses, whereas catecholamines (e.g. epinephrine, norepinephrine and dopamine)
113 increase heart rate and blood pressure (Rabin 1999; Madden 2006). Thus, the HPA/HPI axes
114 and epinephrine stress responses are coupled with metabolism and metabolic rates, as both
115 increase the body's ability to mobilise energy for acute challenges.
116
117 GCs have been used as an indicator of stress, commonly under the assumption that elevated
118 concentration compromises health and ultimately fitness (Millspaugh & Washburn 2004). The
119 HPA response is an adaptive response that allows individuals to respond to acute challenges,
120 however, the relationship between the HPA axis, GCs and fitness is not straightforward
121 (Moberg 2000). There are pronounced species differences in primary GC pathways: cortisol is
122 the primary GC in primates, carnivores and ungulates, whereas corticosterone is the main GC
123 in rodents, birds and reptiles (Touma & Palme 2005). Within these pathways, metabolism and
124 excretion patterns differ between species, as such care must be taken to accurately measure
125 glucocorticoid responses (Palme 2019). Additionally, there is limited and inconsistent
126 evidence for the relationship between GC levels, GC reaction potential and individual fitness
127 (Breuner *et al.* 2008; Bonier *et al.* 2009) as it may be context dependent, such that during good
128 conditions a high GC responsiveness is associated with poor survivorship and recruitment,
129 whereas during poor conditions the relationship may be reversed as individuals in poor condition
130 may be unable to mount significant GC responses (Blas *et al.* 2007). Prolonged or chronic stress
131 (Linklater *et al.,* 2010) is thought to result in the malfunction of the HPA axis (Franceschini *et*
132 *al.,* 2008), leading to chronically elevated or depressed GC concentrations (Linklater *et al.,*
133 2010). This dysfunction of the adaptive allostatic system due to chronic activation, or failure
134 to respond, is termed 'allostatic overload' and can lead to loss of condition, immune suppression
135 and disease. In human studies, the concept of chronic suppression of the HPA axis, or 'adrenal
136 fatigue', has been widely questioned and even dismissed as a phenomenon (Cadegiani & Kater
137 2016), so more evidence is required to substantiate its relevance to animal stress physiology
138 and health. Therefore, to capture the causal impact of environmental challenges on fitness,

we need to move beyond just GC markers to fully understand the fitness impacts on a range of downstream physiological responses.

A second key neuroendocrine pathway that offers potentially useful biomarkers is the hypothalamus-pituitary-thyroid (HPT) axis, which regulates growth, development and metabolism, and helps to maintain a positive energy balance which is fundamental to fitness. Under stable physiological conditions, the HPT axis maintains energy homeostasis, but under challenges, such as extreme temperatures or limited food availability, the HPT up or down regulates metabolic rate by changing the amount of circulating thyroid hormone (Costa-e-Sousa & Hollenberg 2012). When energy intake decreases during fasting or times of calorie deficit, or when external temperatures are very high, circulating thyroid hormone levels decrease. Conversely, in order to maintain core body temperature, for example in response to cold stress, thyroid hormone concentrations increase to upregulate metabolism. These opposing responses to thermal and nutritional challenges can lead to a metabolic trade-off that can be difficult to interpret (Cristóbal-Azkarate *et al.* 2016). There have been fewer studies that utilise thyroid hormones as biomarkers to assess the impact of environmental factors on fitness than those that use GCs. However, there are established assays to measure them non-invasively and they can be an important biomarkers of condition, metabolic state, and therefore energy balance (Behringer *et al.* 2018).

Downstream 'secondary' stress indicators

"Secondary stress responses" are the downstream pathways impacted by HPA/HPT activation such as metabolism, thermal regulation, reproduction and immunity (Sopinka *et al.* 2016). The HPA axis has many cascading effects on physiological pathways that offer potential biomarkers. A consistent downstream impact of HPA activation is in the suppression of reproductive hormones. Androgens, a class of steroid reproductive hormones secreted by the testes, ovaries and adrenal glands, are responsible for the development of secondary sexual characteristics and male behaviour. Androgens and costly secondary sexual characteristics can be suppressed in response to elevated GC production (Folstad & Karter 1992) caused by disparate stressors such as food limitation (Lynn *et al.* 2015), immune (Boonekamp *et al.* 2008) and psychological challenges (Nargund 2015). Similarly, female reproductive hormones, such as progesterone, can be reduced under adverse environmental conditions, leading to lower fertility and increased risk of pregnancy termination (Arck *et al.* 2007). Mineralocorticoids, particularly aldosterone, control water retention, electrolyte balance and blood pressure and are released in response to HPA activation. Increased

production of mineralcorticoids is implicated in cardio-vascular disease (Kubzansky & Adler 2010). Corticosterone is an intermediary in the production of aldosterone such that their pathways are interconnected and the causes and consequences of changes in corticosterone levels may need to be considered in the context of the full pathway.

Inflammatory and immune responses are also molecular indicators of physiological challenge or stress (Sopinka *et al.* 2016; Madliger *et al.* 2018; Celi *et al.* 2019). Immunoglobulins, or 'antibodies' (e.g. IgA, IgG, IgM), form a critical part of the immune response by recognising, binding to and neutralising antigens, such as bacteria or viruses (Schroeder Jr & Cavacini 2010). Faecal antibody assays have been used to measure the immune response to parasites (Watt *et al.* 2016), which in turn correlate with survival (Sparks *et al.* 2018). Another way to evaluate downstream physiological responses to stress as well as pathogen and parasite challenges is to evaluate inflammation markers. Calprotectin and lactoferrin are inflammation markers that limit bacterial growth (Mao *et al.* 2012) and are used to diagnose inflammatory bowel disease in humans (Van Rheenen *et al.* 2010). Such biomarkers gaining traction in human clinical practice have untapped potential for use in wildlife monitoring.

Oxidative stress is another secondary stress response that indicates that challenges have pushed the body into allostatic overload. Increased metabolism results in the production of chemically reactive metabolic by-products known as reactive oxygen species (ROS) (Sies 1991). Typically, ROS are removed from the body by antioxidants, but if they are generated in excess, oxygen radicals build up and bind to a range of biological molecules. This results in cellular and DNA damage, reduced defence mechanism and accelerated aging (Finkel & Holbrook 2000). Chronically elevated GC production is associated with oxidative stress across species (Costantini *et al.* 2011). Additional biomarkers that are associated with short-term and long-term responses to external challenges and stressors are blood pressure, haematocrit levels, heart and respiratory rate and white blood cell counts (Sopinka *et al.* 2016; Madliger *et al.* 2018).

Whole organism or tertiary responses

Physiological changes, from primary to secondary responses, interact in responses to environmental challenges to culminate in whole organism responses. Physiology and behaviour are inextricably linked and it is difficult, if not impossible, to study one without taking into account the other (Cooke *et al.*, 2013). Behavioural changes are a whole-organism indicator to stressors as behaviour responds to multiple physiological pathways (Sopinka *et*

al. 2016) and can act as a first-line of defence against the onset of a stressor (Cameron and Schoenfeld, 2018). All species show some behavioural flexibility, which is a special case of phenotypic plasticity, that can help individuals respond to environmental and social stressors without requiring expensive and inflexible morphological investment (West-Eberhard 1989; Ghalambor *et al.* 2010). Human impacts, however, can disrupt the normal behaviour of animals (Wong & Candolin 2015) and therefore deviations from, or limited expression of, behavioural 'norms' can function as indicators of vulnerability or as indicators of population responses to challenge.

Organisms must maintain a positive energy budget (New *et al.*, 2013) and responses to stressors often incur an energetic cost, which can compromise other investments, such as reproduction or growth (Christiansen *et al.* 2013). In response, an organism can mobilise energy reserves, down regulate metabolism or change its behaviour to increase energy availability by increasing foraging rate, feeding time or travel distances (Reneerkens *et al.* 2002). For example, human winter recreational activities disturb black grouse (*Tetrao tetrix),* increasing allostatic load and causing the birds to increase feeding times and daily energy expenditure by >10% (Arlettaz *et al.* 2015). Sustained negative or positive energy balances will affect the condition of an individual over time. These long-term changes in energy balance can be detected by evaluating changes in body condition scoring, as the loss of muscle and fat reserves suggests a negative energy budget. Body condition scoring is routinely used in the management of wild mammals and standardised schemes have been developed for species including black rhinos *(Diceros bicornis)* (Reuter & Adcock 1998) and African buffalo *(Syncerus caffer)* (Ezenwa *et al.* 2009).

Habitat selection and use can be modulated by, and interact with, physiology. Spatial variation in resources, predation, pathogens and disturbance can drive patterns of space use. For example, ''landscapes of fear" and "landscapes of disgust" models examine how individual space use decisions depend on predation, disturbance or disease risk (Laundré *et al.* 2001; Gallagher *et al.* 2017; Weinstein *et al.* 2018). Landscapes of disgust model how animals can lower parasite risk by moving further away from contaminated faeces (Garnick *et al.*, 2010). Similarly, landscape of fear model habitat shifts due to predation or disturbance that are associated with compromised diet quality and foraging efficiency (Cowlishaw 1997; Barnier *et al.* 2014). Such models can be integrated to create landscapes of stress, where physiological or behavioural trade-offs can be directly incorporated into population or habitat use models

(Koprivnikar & Penalva 2015). For example, brown bears (*Ursus arctos)* near human settlements have lower heart rate variability, a cardiovascular indicator of stress, and they move further during increased human activity, which is expected to have an energetic cost (Støen *et al.* 2015). Disturbance, predation and disease changing an organisms' habitat selection may, therefore, provide an unexpected cause of poor fitness.

Social interactions between individuals are modulated by, and impact on, physiology (Gersick and Rubenstein, 2017; Seebacher and Krause, 2017). Social network analysis (SNA) can quantify the *structure* of a population and the position of individuals within those structures using association, grouping, and space use (Borgatti *et al.* 2009). These networks can reveal how individuals respond to pressures such as resource limitation (Henzi *et al.* 2009; Brent *et al.* 2013; Stanley *et al.* 2018), demographic imbalance or predation (Hasenjager & Dugatkin 2017). Personality, hormones and behaviour can interact in predictable ways which can impact on population dynamics and fitness. Social instability is associated with long term patterns of elevated GCs in spotted hyenas *(Crocuta crocuta)* (Van Meter *et al.* 2009), Barbary macaques *(Macaca Sylvanus)* (Edwards *et al.* 2013) and olive baboons *(Papio anubis)* (Sapolsky 1992). Chronically-depleted GC metabolites are associated with reduced social interactions (Barik *et al.* 2013) and increased aggression (Haller *et al.* 2004). House finches (*Haemorhous mexicanus)* with higher baseline corticosterone are more likely to exhibit exploratory behaviour and more exploratory individuals have a higher social network degree and are more likely to be dispersers (Moyers *et al.* 2018). Thus, modelling perturbations to networks in response to environmental change, stressors and interventions should be a more common conservation tool, especially in conjunction with physiological biomarkers.

Responses of symbiotic and parasitic species

Within the animal gut, diverse microbial communities and their genes - referred to collectively as the gut microbiome - perform key functional roles in the host and contribute significantly to host health (Sommer & Bäckhed 2013; Gilbert *et al.* 2018). An imbalance of the microbial community, known as dysbiosis, can reduce digestive efficiency, increase inflammation and susceptibility to infection (Dethlefsen *et al.* 2007; Amato *et al.* 2013; Gilbert *et al.* 2016). Microbiome communities are influenced by a range of factors such as habitat, diet, social network properties and climatic conditions (Trevelline *et al.* 2019). Diet changes can lead to the loss of key microbiota, which negatively impacts gut function (Borbón-García *et al.* 2017). Furthermore, primary and secondary indicators of stress such as GCs modulate the

microbiome (Noguera *et al.* 2018). Reproductive performance has been associated with microbiome composition (Antwis *et al.* 2019), as has cellular and molecular stress biomarkers of cellular inflammation (Walshe *et al.* 2019).

Infectious wildlife disease is an increasingly alarming threat to biodiversity. Widespread human impacts impact population health and performance through immunosuppression caused by stress. Small populations additionally have decreased genetic diversity, lowering population resilience to novel disease (Brearley *et al.* 2013). Although there is limited causal evidence between human impacts, stress and disease occurrence, it is widely assumed that stress may be a major cause of increased susceptibility to wildlife disease. Widely applicable techniques such as qPCR (Dale *et al.* 2016) assays for diagnoses of pathogens could be paired with biomarkers or modelling (Lachish *et al.* 2012) at the landscape level to further uncover the relationships between physiology, behaviour, human impacts and disease.

Parasite species richness negatively impacts survival and fecundity, which together can impact on population dynamics (Hudson *et al.* 1998; Hillegass *et al.* 2010). Steroid hormones, including GCs and androgens, affect immune function and are affected by parasite loads (Klein 2004), however, the evidence for direct relationships between elevated GCs and parasite burden is limited. Gastrointestinal nematode communities, or the nemabiome, have the potential to influence resistance and susceptibility to other infecting species (Supali *et al.* 2010). Thus, heavy parasite burdens have significant impacts on fitness proxies. However, parasite infections are not universally harmful, removing helminths induces a strong inflammatory response (Walshe *et al.* 2019) and can potentially trigger autoimmune diseases (McKay 2009).

Integrating environment, biomarkers and fitness

In sum, environmental stressors can impact multiple biological pathways with cascading effects impacting on behaviour, immunology, energetics, life history and ultimately individual and population fitness (Figure 1) (Raab *et al.* 1986). Multiple stressors can act independently or in tandem causing additive, synergistic or antagonistic effects (Beldomenico & Begon 2010; Todgham & Stillman 2013). Given the complexity of physiological reaction to stress and the challenges in identifying how stressors impact on individuals and populations, using one biomarker from a single pathway can give an incomplete or even misleading picture of the connection between stress and fitness. However, using a suite of biomarkers can provide

information about how the different pathways interconnect and impact fitness in different environments (Figure 2, Table 1). Despite calls for this integration, few studies employ multi-tool approaches to evaluate the impact of stress on multiple physiological pathways. In a review of physiological studies, only 26% of studies used multiple markers from different pathways and only 52% used multiple markers from the same pathway (Madliger *et al.* 2018). Studies which investigate stressors, physiology and demography together are scarce (Beehner & Bergman 2017) but a few do exist (Table 1; Figure 2-5) (Arlettaz *et al.* 2015; Lea *et al.* 2018). Integrating different biomarkers give a much more complete picture of how extrinsic threats impact individuals and lead to population changes.

A range of subdisciplines (e.g. immunology, behaviour, physiology) all study the reaction of an organism or population to a stressor. These sub-disciplines are usually studied in isolation, but they are all studying the same thing: a stress response manifesting through a variety of different "pathways" in the organism. Moreover, these markers essentially have two functions: to measure positive indicators of health and fitness and to measure negative indicators of allostatic load. In essence, the goal is to understand the *functional condition*, or the net positive and negative states of an organism, as a proxy or predictor of fitness. In order to encourage research that integrates different biomarkers in this way, we propose a multiplex pathways framework, where pathways are identified by metabolic footprints, or secretory products, of intra-cellular metabolism (Hollywood *et al.* 2006). These footprints from different metabolic or biological pathways can be combined with behaviour, body condition or disease burden biomarkers to provide a holistic picture of health and condition. We believe that developing this approach would be extremely valuable in ecology and conservation.

Applying functional footprints to ecological theory

Across a species' distribution, populations will vary in terms of growth rates, density and population size. Although the theoretical bases and causes of variation in performance are well established, the underlying physiological mechanisms are poorly understood. Evaluating variation in physiological footprints within and across populations has untapped potential to determine species range boundaries, ecological tolerances and population viability and predict large-scale species' responses to environmental change (Chown & Gaston 2008; Gaston *et al.* 2009). Until recently, large scale macro-physiological/ecological studies have

348 been impractical for many species, especially as characterising interactions between multiple
349 stressors and physiological responses is challenging (Todgham & Stillman 2013). However, an
350 increasing breadth of validated biomarkers tied together with complex data modelling has
351 made multi-level assessments increasingly tractable. Here, we introduce two hypotheses, the
352 *Functional Marginality Hypothesis* and the *Functional Recovery Hypothesis* to illustrate how
353 physiological pathways can identify mechanisms driving large-scale population and
354 distribution patterns, both in the present and in predicting future responses, and aid the
355 design and assessment of conservation interventions.
356
357 *Functional Marginality Hypothesis: environmental change, range dynamics and ecological*
358 *retreat*
359
360 Species physiological and behavioural tolerances to ecological gradients determine their
361 realised niche, range dynamics and geographic limits (Pearman *et al.* 2008; Sexton *et al.* 2009).
362 Across a species' range, ecological gradients and fitness will vary from optimal habitats where
363 conditions and resources permit maximum birth rate and minimum death rate (Holt 2009), to
364 marginal habitats where reproduction and survival are comparatively low (Kawecki 2008).
365 Better quality environments with a high potential rate of population growth become 'source'
366 populations which supply individuals, through emigration, to non-sustaining 'sink' populations
367 in marginal habitats (Pulliam 1988). These marginal populations may be completely reliant on
368 immigration from source populations for their viability (Pulliam & Danielson 1991). The
369 simplest species response models assume that fitness proxies follow a unimodal distribution
370 with the highest densities and growth rates in the centre of a range and lowest at the
371 periphery (Guo *et al.* 2005). Although easy to model, a Gaussian response curve may not
372 accurately reflect species tolerances, especially near range limits (Sagarin *et al.* 2006).
373 Although, sources may be more common near the centre of the range and sinks more
374 common near the periphery, ranges are likely to have a complex topology of sources and sinks
375 interspersed with areas of unsuitable habitat.
376
377 Species distribution models (SDMs), such as habitat suitability models or bioclimatic envelope
378 models, are important tools for modelling species potential ranges (Guisan & Zimmermann
379 2000). These models relate known or inferred species occurrence to environmental variables
380 in order to explain or predict where a species is likely to occur. SDMs are widely used in
381 macro-ecology, evolutionary biology, conservation and management (Jiménez‐Valverde *et*

382 *al.* 2008). A major assumption of many SDM models is that extant populations are found in
383 optimal or 'good' habitats and species are absent from 'poor' habitats (Braunisch *et al.,* 2008),
384 which may not be true, especially in declining or expanding species. Phenomena such as
385 ecological traps (Hale & Swearer 2016), niche denial (Kinnear *et al.* 2002) and ecological
386 refugees (Kerley *et al.* 2012) can lead to species becoming confined to sub-optimal
387 environments. Failure to identify marginal habitat traps within heterogeneous landscapes can
388 over-estimate the extent of suitable habitat (Braunisch *et al.,* 2008). Similarly, not identifying
389 unoccupied suitable habitat can lead to underestimation of potential range. These errors can
390 be minimised using mechanistic models where the niche, or physiological tolerance, is defined
391 from experimental or field based data (Kearney 2006). In a mechanistic model, regions with
392 characteristics that negatively impact physiological state enough to suppress survival, growth
393 or reproduction to unviable levels are removed, or down weighted, in the final distribution
394 (Kearney & Porter 2009).
395
396 While mechanistic SDMs are generally considered to be more ecologically meaningful
397 (Kearney & Porter 2009), they have previously been perceived as impractical or unfeasible for
398 most species and geographical regions (Guisan & Zimmermann 2000). However, using
399 multiple physiological biomarkers from natural populations, rather than controlled
400 experiments, can allow a mechanistic approach across large geographic regions. In a changing
401 world, there is a need for comparative longitudinal, broad spatial-scale data, which makes
402 physiological indicators promising tools as they can improve the accuracy and effectiveness
403 of SDMs and the resulting conservation interventions (Evans *et al.* 2015). Physiological
404 biomarkers can be used to monitor entire freshwater ecosystems and predict future
405 population-level changes in response to habitat management (Lennox *et al.* 2018).
406 Additionally, as mechanistic models are independent of current ranges, extrapolation to
407 unoccupied habitats is possible, which allows for better estimation of suitable range,
408 colonisation and invasion potential under different environmental change scenarios.
409
410 If physiological tolerances determine range boundaries and regulate densities (Lee *et al.*
411 2009), biomarkers can reveal how individual physiology and behaviour vary with ecological
412 and demographic factors. These can be applied at large spatial and temporal scales to
413 mechanistically link environmental variation with individual and population-level
414 performance (Buckley *et al.* 2010). For example, a number of macrophysiological studies using
415 biomarkers used a mechanistic approach to evaluate how environmental change impacts on
416 aquatic biota at the population level (for review, see Colin *et al.,* 2016). The *Functional*

Marginality Hypothesis proposes that the response functions of related biomarkers (e.g. glucocorticoids, immune function, reproduction and energy balance) covary and are synergistic at both the upper and lower ends of potential population performance (Figure 5a). In optimal habitats we expect an increase in 'positive' biomarkers such as body condition, reproductive hormones and metabolic rate, and a decrease in 'negative' biomarkers such as parasite burden, glucocorticoids, microbiome dysbiosis and oxidative stress. The net effect of 'functional condition' on population performance can be compared using a model selection approach. This predictive framework can be applied to model species' ecological tolerances using biomarker responses over space and time. For example, we would predict range-wide habitat degradation and climate induced changes would cause a shift upwards in negative biomarkers and a shift downwards in positive biomarkers across the range, or a net reduction in functional condition with a shift of populations from sources to sinks. Conversely, ecological retreat would cause a directional shift towards suboptimal marginal habitats. In this case, we would expect a truncated distribution of markers towards less optimal states and a higher proportion of sink populations across the remaining range (Figure 5c).

Environments change due to natural processes which currently are accelerated by anthropogenic impacts, including climate change and land use change. Marginal populations can be identified by 'poor' condition on multiple biomarkers and ground-truthed by evaluating spatial variation in reproductive performance. Physiological and behavioural biomarkers have been used to help identify 'refugee' populations (Figure 3) (Lea *et al.* 2016; Lea *et al.* 2018), through evidencing physiology, behaviour and gut health gradients across realised niche space. This framework can be used to test alternative models of range retreat: if environmental change causes species to retreat into ecological utopias, then functional biomarker footprints should be consistent with biomarker profiles exhibited in optimal habitats or source populations. Conversely, if range contraction is driven by retreat into suboptimal refuges from encroaching ecological threats, then biomarker footprints in the contracting edge will have high levels of negative biomarkers, and poor functional condition, compared to individuals from previously occupied 'source' habitats (Figure 5c). For example, habitat conversion leads to dietary shifts, time budget changes and changes in gastrointestinal microbial communities in common vampire bats (*Desmodus rotundus)* and the relative abundance of some microbiota taxa was associated with innate immune function (Ingala *et al.* 2019). However, to fully employ biomarkers to predict resilience, we require a greater understanding of individual physiological responses, down-stream fitness consequences and population-level responses across ecological gradients (Bonier *et al.* 2009).

The predictions stemming from this hypothesis are not only useful for studying current ecological patterns, but a number of key studies have used a functional footprint approach to predict resilience, or lack thereof, in populations of conservation interest. For example, ectotherms in high altitude environments display slower growth rates and longer times to reach sexual maturity compared to ectotherms at lower altitudes (Morrison & Hero 2003). When the threats at higher and lower altitudes are of equal intensity and effect, such as risk of adult mortality, higher altitude ectotherms have greater extirpation rates than lowland ectotherms of the same species (Muths *et al.* 2011). A number of physiological traits in marine biota, such as heart function, action potential generation and protein thermal stability, exhibit adaptive variation in response to changes in temperature across vertical zonations along an intertidal gradient (Somero 2002). Sea level changes due to climate change will lead to intertidal species experiencing extensive periods emersed and spending more energy on thermal regulation. This can be used to predict which species and populations will be most severely affected by future changes in environment. In sum, the *Functional Marginality Hypothesis* has predictive power to investigate range limits, dynamics and contraction or shift due to ecological threats and climate change and population performance.

Functional Recovery Hypothesis: evaluating colonisation, reintroductions and translocations

As human impacts have extensively changed and degraded habitats, conservation efforts often try to restore habitats or populations to reflect a historical state or ecological baseline. A mechanistic approach can provide the evidence about how best to restore or manipulate degraded systems and how to establish whether an intervention has had the desired response (Hobbs *et al.* 2014). We define *functional recovery* as when an intervention or restoration results in physiological and behavioural footprints returning to a profile similar to its pre-perturbation state or to a profile similar to that associated with an undisturbed viable population. In the *Functional Recovery Hypothesis,* we would predict following an intervention where the target population is experiencing problems, negative biomarkers will remain higher, and positive biomarkers will remain lower, than pre-intervention levels (Figure 2d). Conversely, a positive intervention will allow biomarkers to return to the same level as an equivalent undisturbed viable population.

One serious issue is that we often have limited evidence for appropriate baselines such that there is a degree of speculation and guesswork about where species should occur. Where we

have limited evidence of historical baselines in terms of community composition, population density or appropriate habitat, we need new metrics to deem whether an intervention has been successful. For example, large-scale reintroduction programmes are taking place to restore species throughout their historical ranges (Alagona *et al.* 2012), however, reintroduction programs have proven to be very challenging (Duarte *et al.* 2009; Hobbs *et al.* 2009). The *Functional Recovery Hypothesis* provides a new metric by which to test intervention success. For example, monitoring pregnancy rates of vertebrates before and after restoration or policy implementation can provide information about whether reproduction has improved as a result of interventions (Pallin *et al.* 2018). Assessment of stress hormones pre- and post-intervention could also evidence efficacy (Cooke & Suski 2008). These tools give important insight into the performance consequences of restoration biology, colonisation and reintroduction.

Colonisation and extirpation are natural processes that have been accelerated by human activities. Colonisation of a species may have unintended consequences on the existing system (Armstrong & Reynolds 2012). For example, recent recolonization of the Central European Lowland by grey wolves (*Canis lupus)* impacted the endoparasitic species richness within the population (Lesniak *et al.* 2017) and increased parasite loads within their prey species (Lesniak *et al.* 2018). Predicting colonisation potential and extirpation risk for species, due to the establishment and spread of other species whether naturally or due to human action, is key to ecological processes and conservation efforts. The *Functional Recovery Hypothesis* predicts that species will be able to colonise habitats where their relative biomarker score indicates they can maintain a viable population. Species negatively affected by the colonisation of another species would have higher negative biomarker levels compared to viable non-colonised populations. This hypothesis has applicable predictive power for establishment, spread and impact of invasive species (Figure 5b) (Phillips & Shine 2006). For example, declining red squirrel (*Sciurus vulgaris*) populations under competition from the invasive grey squirrel (*Sciurus carolinensis*) have higher GC levels and fall to pre-invasion levels once the invasive grey squirrel is removed (Santicchia *et al.* 2018).

Many recolonisations are mediated and carried out consciously by humans. However, lack of evidence about habitat suitability can lead to reintroductions into marginal areas or inadequate management of existing populations. For example, a stitchbird *(Notiomystis cincta)* population was reintroduced onto a predator-free island which lacked adequate food

521 sources for the species (Armstrong & Perrott 2000; Armstrong *et al.* 2002). The functional
522 condition approach can allow the success of reintroductions to be monitored so that problems
523 can be rectified. For example, comparing thyroid and GC levels of animals pre- and post-
524 release could have monitored energetic states in the new habitat (Box 3), which may have
525 prompted supplementary feeding before the population was extirpated.
526
527 Translocation success is linked to stress responses and resilience to change, which
528 occur during the translocation event and the establishment phase immediately after release
529 (Dickens *et al.,* 2010). However, the impact of translocation on behaviour and stress
530 physiology has been studied in few species and in few habitat types (Fischer & Lindenmayer
531 2000; Germano & Bishop 2009; Seddon *et al.* 2012; Tarszisz *et al.* 2014). Using the *Functional*
532 *Recovery Hypothesis*, we would predict after a recovery phase, positive and negative
533 biomarkers should return to pre-translocation levels. For example, translocation stress in
534 African elephants revealed increased GC levels after movement. Subsequently, GC levels only
535 returned to pre-translocation levels 23 days after the elephants were able to migrate back
536 into their original home range (Viljoen *et al.,* 2008). Whether the faecal GC levels would have
537 returned to normal without the behavioural coping of returning to the pre-translocation site
538 is unknown but translocated animals have been known to lose weight (Dickens *et al.* 2010).
539 Furthermore, given that there are interactions between GCs, microbiome (Noguera *et al.*
540 2018) and parasite resistance (Nair *et al.,* 1981), translocation stress may result in long-term
541 changes to physiology through indirect means.
542
543 Importantly, small perturbations that are hard to study using traditional methods can be
544 detected using the functional footprints approach. These can result in population wide
545 behavioural changes and potentially have drastic physiological consequences due to additive
546 effects. For example, wild great tits (*Parus major)* increase the number of social associations
547 and overall network connectivity in response to the removal of a small proportion of
548 individuals (Firth *et al.* 2017). If small successive perturbations occur within short intervals
549 where the species cannot complete their functional recovery, effects may be cumulative and
550 negative consequences occur. Long-term social instability has been associated with increased
551 GC concentration in multiple species (Van Meter *et al.* 2009), which may predispose
552 individuals to chronic stress (Nuñez *et al.* 2014). Understanding species' ability to
553 physiologically and behaviourally adapt to changes in its environment will help increase the
554 number of successful reintroductions and translocations (Dickens *et al.* 2010).
555

Conclusion

In recent decades, much research has been carried out to develop biomarkers which provide an indication of the impact of the environment on the physiological and behavioural state of an organism and ultimately on fitness. This is a difficult task as physiology is extremely complex. Physiological footprints are the result of multiple interconnecting pathways, which can respond to the same stressors and interact with each other, making the change in a single biomarker difficult to relate to fitness. We propose that using complementary and integrated biomarkers to highlight key changes in footprints and pathways would be a major advance for large scale ecology and conservation. Such a physiological toolkit can help to assess these pathways at individual, population and landscape scales in order to investigate the causes of poor individual health and changes in survival and reproduction. This information can then help to uncover the causes of distributional limits and predict future changes, estimate resilience of populations to novel threats, assess the efficacy of conservation efforts, and reveal macro-ecological trends and processes. This footprints and pathway approach provides conservation biologists and practitioners the ability to produce evidence for the causal mechanisms underlying conservation problems and macro- or evolutionary ecologists the ability to investigate the physiological mechanisms underlying long-term and large-scale processes. Advances in these fields can contribute towards the calls for evidence-based conservation and help to alleviate the threat of species extinctions and ecological collapse.

Acknowledgements

The authors would like to thank anonymous reviewers for constructive feedback that significantly improved this manuscript. We thank Danielle Hinchcliffe for discussion about the approach and framework of this manuscript. SS is supported by a Royal Society URF. NH and JB are both supported by the NERC EAO DTP program.

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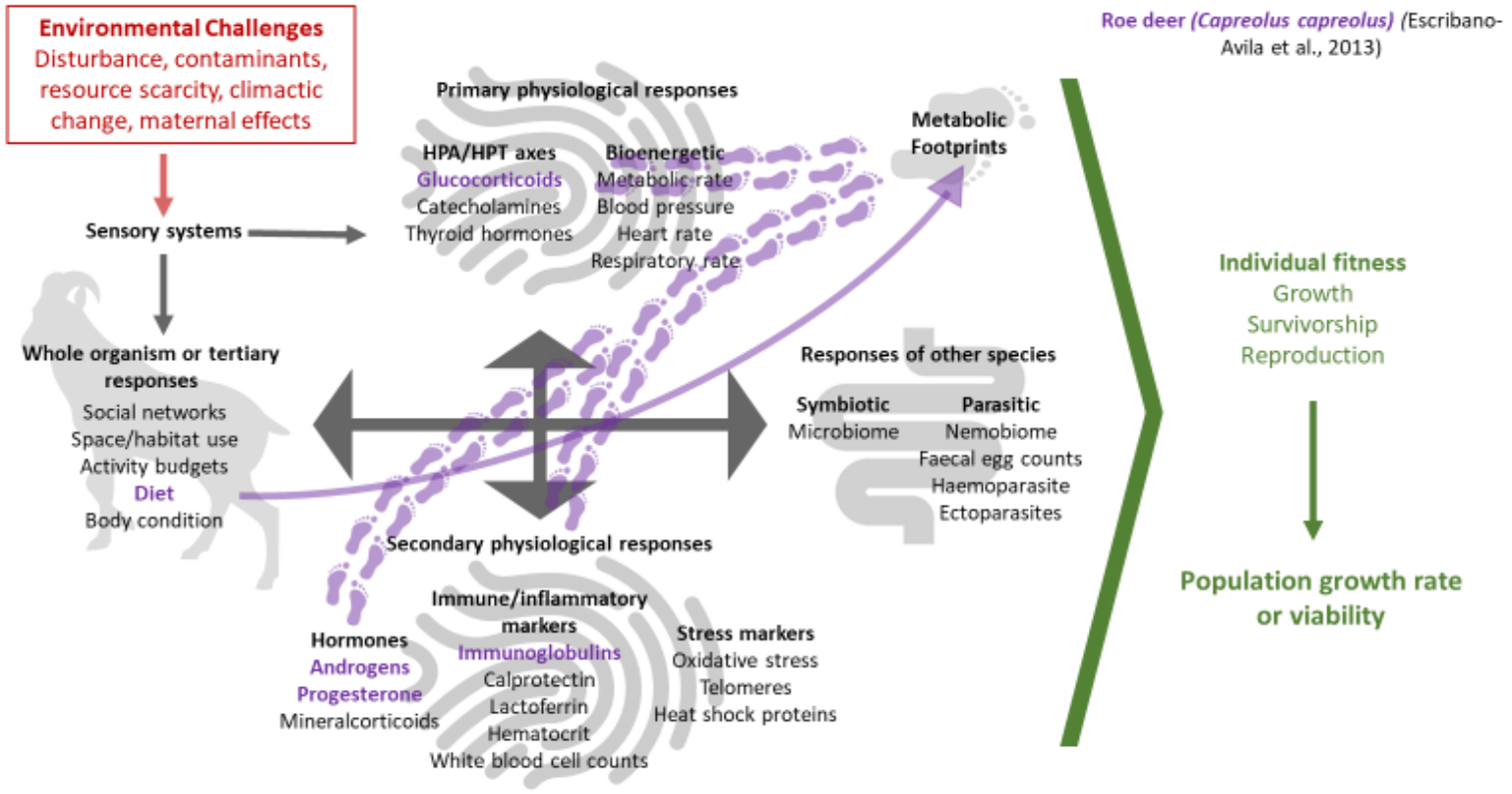

Figure 1. A conceptual diagram showing the different biomarkers available that be integrated into study that uses the footprints and pathway approach. The biomarkers in purple were used by (Escribano-Avila et al., 2013) in their study of the impact of resource scarcity on reproduction.

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Figure 2: Life at the extremes of the ecological niche

Savannah-mosaic habitats are thought to represent the physiological limit of the species fundamental niche for chimpanzees (*Pan troglodytes*). Wessling and colleagues (2018) hypothesised that if these habitats are marginal; chimpanzees living in these habitats should demonstrate physiological consequences of the living at the edge of their potential ecological niche.

Wessling *et al, 2018* compared seasonal variation in physiological responses to climatic and ecological factors in two populations of Chimpanzees: those inhabiting Fongoli, a savannah-mosaic habitat at the margins of the chimpanzee range and Taï National Park, a lowland rainforest centrally located within the West African chimpanzee subspecies (*P. troglodytes verus*) range. Wessling *et al* compared the urinary biomarkers of creatinine, c-peptide and stress to investigate dehydration, energetic status and stress level respectively. Fongoli Chimpanzees experienced higher physiological seasonal costs in the form of elevated cortisol levels and both populations displayed limitations due to dehydration. Therefore, species range limits were implied to be formed due to the physiological tolerance of chimpanzees in Fongoli to thermoregulation. In other words, the excessive arid and hot environmental conditions limit an individual's ability to maintain homeostasis.

Image: IUCN Red List

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Figure 3: Ecological refugees: conservation in marginal habitats

In addition to looking at resilience, we can also use these tools to evaluate individual health and reproduction as a function of other challenges, such as parasite burden, demographics, territory or range quality, inbreeding coefficients, and age. The nature of macroecology produces an intertwined mesh of multiple disciplines, which may co-vary and interact to form the overall individual and population health. For example, Lea *et al,* (2018*)* used faecal markers glucocorticoids and androgens, to measure chronic stress and male physiological status respectively, in the cape mountain zebra (*Equus zebra zebra*). The cape mountain zebra show great variability in fecundity and glucocorticoid concentrations across protected areas leading to poor population growth in many areas (Lea *et* al, 2016). Lea *et* al, (2018) found glucocorticoids were elevated in individuals living in low-quality habitat and testosterone concentrations were higher in groups with higher numbers of males to females. These results linked individual physiological biomarkers with environmental and demographic variables respectively. As such, the Cape Mountain Zebra is now regarded as a partial refugee species (Lea *et al.* 2016) *i.e.* is maintained in a sub-optimal population across a proportion of its protected areas and range. Allowing for a set of non-invasive tools will help to elucidate more of processes and interactions tying aspects of biotic and abiotic factors together.

Image: Dr Jessica Lea

Figure 4: Using biomarkers to assess food limitation and impacts of disturbance of a critically endangered species across its' species range

Hawaiian monk seals have experienced declines since the 1950s and are listed as Critically Endangered on the IUCN Red List of Threatened Species (IUCN, 2010). It was hypothesised that the major cause of decline across the species' range was poor survival of juveniles due to limited resources (Harting, 2002). Gobush *et* al, (2014) assessed the impacts of food limitation and human disturbance on the physiology of the monk seals across their range; specifically, on the main Hawaiian Islands (MHI) where the seal population was growing and the Northwestern Hawaiian Islands (NWHI), where the population was in decline. Gobush *et al*, found declining subpopulations exhibited chronic elevation of fGCMs and low fT3, especially in immature individuals. fGCMs were highest at French Frigate Shoals (a NWHI site) while fT3 was relatively low indicating, a possible signal of food limitation in this population. Populations with higher fGCM levels had, on average, poorer survival rates and lower intrinsic population growth rates. Comparing fT3 concentrations across reserves suggested that although there may be adequate food sources, populations varied in their physiological cost to obtain these food sources. Furthermore, disturbance appears to impact monk seal physiology less than other stressors. Anthropogenic disturbance from the residents and tourists did not appear to impact the physiological condition of the seals or the growth of the populations. Therefore, non-invasive metabolite analysis revealed the potential causes of sub-population decline in a critically endangered mammal while removing other potential causes.

Image: National Geographic

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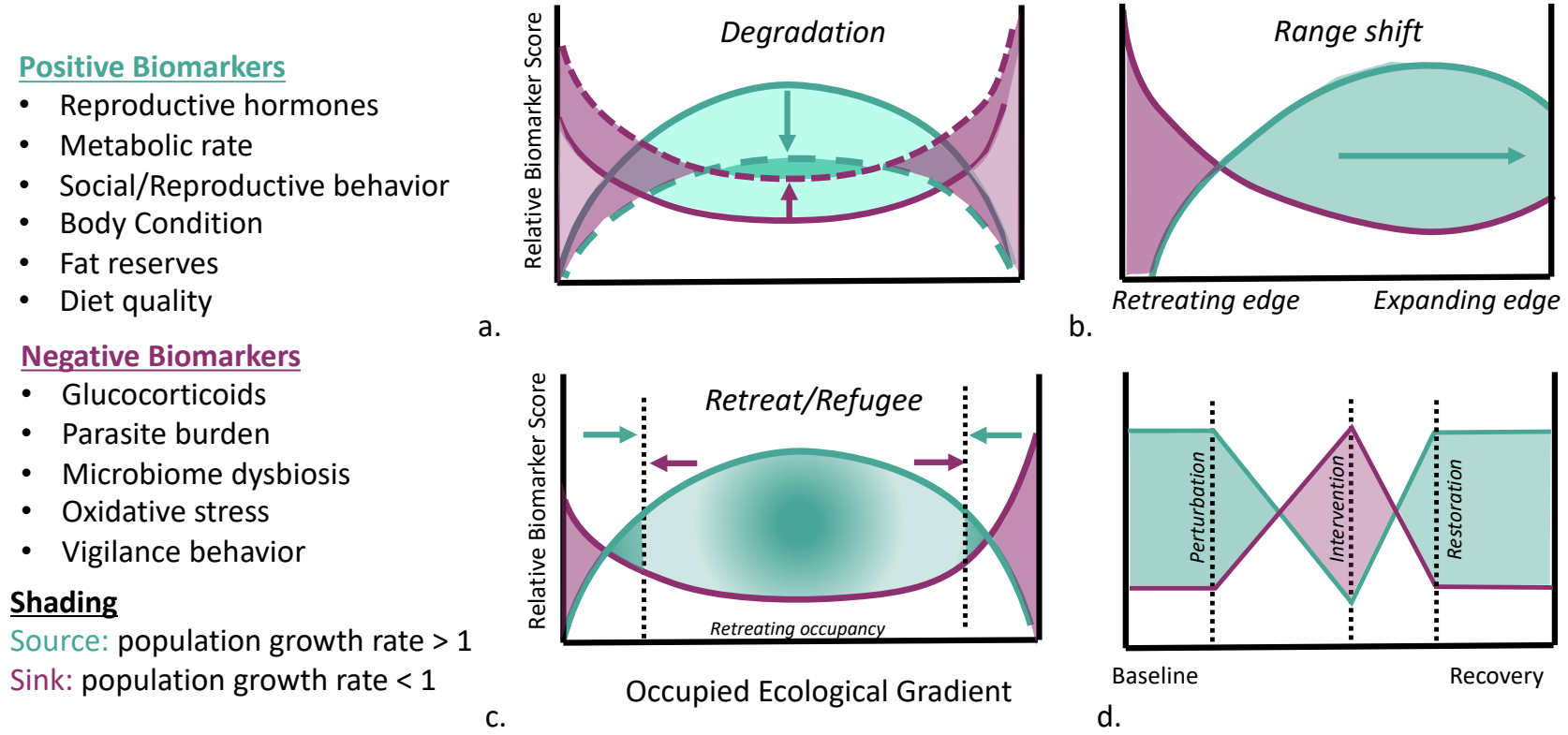

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1066 Figure 5. Conceptual diagram of the *Functional Marginality* (a-c) and *Functional Recovery (d) Hypotheses*. a) Habitat degradation leads to a net decline in
1067 functional condition (balance of positive indicators and negative allostatic load) across occupied habitat resulting in more sink populations and fewer source
1068 populations. b) Range shifts will show an improving functional condition on the expanding edge and declining condition on the retreating edge. c) Refugee
1069 species will demonstrate retreat into areas with lower condition (purple arrows), whereas species retreating into ecological utopias will be associated with a
1070 high mean and low variance in functional condition.

1071 Table 1: Example studies linking physiological challenge with demographic/population consequences. These are relevant examples which demonstrate study design which
1072 incorporates a footprints and pathways approach linking environmental challenge, using multiple biomarkers that response the challenge and the consequence to fitness.
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Species	**Challenge**	**Biomarkers**	**Population/fitness consequences**	**Reference**
Killer whales *(Orcinus orca)*	Fish abundance Vessel density	Feacal GCs Faecal T3	Pregnancy loss	Wasser et al., 2017
African elephants *(Loxodonta africana)*	Rainfall	Faecal GCs Faecal progesterone	Declines in progesterone indicates a decline in reproductive function	Foley et al., 2001
Shetland ponies *(Equus caballus)*	Winter	Heart rate Locomotor activity Thyroid hormone	Field metabolic rate	Brinkman et al., 2016
Damselfly *(Enallagma cyathigerum)*	Predation	Stress proteins O2 consumption Enzyme activity	Growth rates Oxidative stress	Slos and Stoks, 2008
Soay sheep *(Ovis aries)*	Maternal effects Genetic variation	Ig proteins Faecal egg counts	Survival	Sparks et al., 2018
Roe deer *(Capreolus capreolus)*	Primary productivity	Testosterone Progesterone Estradiol GCs Faecal nitrogen IgA	Reproductive condition	Escribano-Avila et al., 2013

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