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STRESS INDICATORS IN FISH

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A fish is chased with a net in an aquarium before being captured, scooped out of the water, and placed in a nearby testing arena. Is it stressed? How can we tell? Are our indicators reliable? Quantification of stress in fish has evolved from the initial development of radioimmunoassays to measure cortisol in plasma to the rapidly expanding suite of genome-based assays. Indicators range from the intracellular to whole-animal level. Expression of heat shock proteins (HSPs) and activity of metabolic enzymes can be paired with straightforward observations of reflexes and survival. Both traditional and emerging indicators have advantages and disadvantages, and their use is

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tissue- and context-specific. Ecological, biological, and methodological factors must be considered when selecting, measuring, and interpreting stress indicators. Inter- and intraspecific, sex, life stage, and temporal differences in physiological responses to stressors can confound confirmation of a stressed state. Despite numerous types of indicators, our understanding of how absolute levels of indicators relate to stressor severity and recovery to date remains limited. How accurately indicators characterize stress in wild populations naturally exposed to stressors is still an evolving discussion. The integration of research disciplines and involvement of stakeholders and user groups will aid in filling these knowledge gaps, as well as the translation of individual-level indicators to population- and ecosystem-level processes.

1. WHY DO WE MEASURE STRESS?

As explained by Schreck and Tort (2016; Chapter 1 in this volume), stress is an inherent component of the life of all vertebrates, including fishes. Measures of stress inform us how effectively a fish resists death and resets homeostatic norms when faced with noxious stimuli. This information is then translated into evolutionary and ecological theory to understand how animals are adapted to, or able to adapt to, future stressors. From a fundamental perspective, measuring stress contributes to our knowledge of carryover effects (eg, O'Connor et al., 2010), parental effects (eg, Sopinka et al., 2014), personality (eg, Aubin-Horth et al., 2012), and life history variation (eg, Pottinger and Carrick, 2001; Ricklefs and Wikelski, 2002). Without classifying, quantifying, refining, and interpreting indicators of stress, the significance and implications of a fish's response to external and internal environmental change would be unclear (Schulte, 2014). For example, without a clear stress indicator, can an animal's response be defined as stress? Can undisturbed animals be characterized as adequate experimental controls? Further, could an environmental change be classified as a stressor? Stress transcends levels of biological organization; measuring stress also serves to link organismal responses to population-level processes (Calow and Forbes, 1998; Fefferman and Romero, 2013) and ecosystem health (Dale and Beyeler, 2001).

From an applied perspective, measuring stress is necessary to determine how the health, performance, and welfare of fishes are being influenced by interactions with humans. For example, assessing stress in captive

populations of fish (eg, hatcheries, farms, aquariums) is often done with a goal of reducing stress to maximize growth and survival. In fact, the empirical study of stress in fishes is rooted, and continues to be prolific, in aquaculture studies that examine how handling, rearing, and transport (Barton et al., 1980; Portz et al., 2006), as well as anesthesia (Iwama et al., 1989; Trushenski et al., 2012), affect captive broodstock health and production efficiency. Foundational mechanistic work quantifying stress was, and continues to be, performed using domesticated species (eg, rainbow trout, *Oncorhynchus mykiss*; tilapia, *Oreochromis* spp.). Stress indicators are now used as objective indices of the welfare status of fish (Iwama, 2007), and to understand the impacts of recreational (eg, Morrissey et al., 2005; Landsman et al., 2011) and commercial fisheries (Marçalo et al., 2006; Raby et al., 2015), as well as laboratory animal husbandry (Brydges et al., 2009; Eaton et al., 2015) on fish performance. There is also increasing interest in optimizing the use of stress indicators to quantify the condition of wild populations (Madliger and Love, 2014). Combining basic and applied motivations to measure stress is especially valuable. Linking evolutionary and ecological underpinnings of stress with measures of stress relevant to industry and conservation practitioners can guide management strategies that effectively take into account fish biology, and facets of human livelihood and culture.

Here, we provide an overview of stress indicators ranging from the cellular to whole-animal level (Section 3). We then outline important considerations when measuring and interpreting these indicators (Section 4), discuss the extension of individual-level indicators to population- and ecosystem-level processes (Section 5), and conclude with avenues of research and novel indicators that warrant further investigation (Section 6). We acknowledge the pioneering syntheses on this topic in S. Marshall Adams' book, *Biological Indicators of Stress in Aquatic Ecosystems* (Adams, 2002), and especially the chapter on physiological and condition-related indicators of stress in fish by Barton et al. (2002). We encourage readers to consult these works for additional insight on stress indicators in fishes.

2. QUANTIFYING STRESS

Approaches to quantifying stress in fish are varied depending on the targeted indicators (Figs. 1 and 2, Section 3). A main distinction in quantification is whether the stress response itself is being measured (ie, the primary response/HPI axis activity) (Gorissen and Flik, 2016; Chapter 3 in this volume), or other physiological, behavioral, or life-history traits (ie,

secondary and tertiary measures; Mazeaud et al., 1977; see Section 3) predicted to change in concert with, or as a result of, stimulation of the HPI axis. Assessing the magnitude of the response that an individual mounts when exposed to an acute or chronic stressor typically utilizes repeated sampling over a period of time. Establishing as accurately as possible baseline/resting levels of the desired indicators is necessary and serves as the first time-point in a series of measurements. Changes in key primary parameters (eg, catecholamines, cortisol) over time are measured to characterize the stress response (see Section 3.2). When chronic stress is quantified, resting levels of primary indicators are measured upon cessation of the stressor exposure (vs levels induced by an acute stressor following the chronic stressor exposure). These established methods for quantifying the stress response, though largely replicated and consistent, represent only one piece of the whole-animal response to a stressor (see Section 3.3).

Experimental assessment of the stress response is not without its challenges. Inability to repeatedly sample individuals due to body size constraints (ie, fish that are extremely large or extremely small), unintentional omission of quantifying peak levels of the indicator based on predetermined sampling intervals, and capture and handling during tissue sampling initiating a secondary mounting of the stress response (Baker and Vynne, 2014), can all limit statistical power and comprise interpretation of data. Habituation during chronic stressor exposures can also skew interpretation (see Section 4.4). Ultimately without measurements of fitness, or proxies thereof, such mechanistic studies are limited in their extension to population-level trends (Calow and Forbes, 1998; see Section 5). Mechanistic studies are, however, essential in building fundamental knowledge of stress, motivating and guiding design of research which focuses on physiological and behavioral processes interconnected with stress, and streamlining future validation of stress (ie, targeting a single poststressor time-point to confirm mounting of the stress response in study animals).

Based on established relationships between stressor exposure, HPI activity, and a range of whole-animal responses (reviewed in Wendelaar-Bonga, 1997; Iwama et al., 1997; Mommsen et al., 1999; Schreck, 2010; Barton, 2002), quantification of stress can refer to quantification of primary, secondary, and tertiary responses that occur following HPI axis activation (eg, gene expression, immune function, metabolism, growth, reproduction, performance, behavior; see Section 3). Quantification of responses typically requires measurement of pre- and poststressor levels of the indicator. Often, the latter response is measured once following stressor exposure. Differences observed pre- and poststressor are treated as an indication of stress. This sampling regime provides a snapshot of how the trait has changed in the

stressor-exposed animal but does not guarantee that maximum response or response recovery is captured. Generally, when responses are quantified, a primary indicator directly related to HPI axis function (eg, plasma cortisol) is also measured prestressor exposure, and at a single poststressor time point. The time-points for primary, secondary, and tertiary stress indicators ideally should be based on preliminary time course sampling using the study species and specific stressor (eg, Pickering et al., 1982; Donaldson et al., 2014). A number of confounds can arise when the time-point at which the indicator will be sampled is chosen arbitrarily. For example, two fish can have the same elevated stress indicator at the sampling time-point, but one individual on a trajectory to death and the other individual on a trajectory to recovery. Further, depending on whether stressors are continuous or sequential (see Section 4.4) it is possible that indicator levels will oscillate throughout an exposure (Schreck and Tort, 2016; Chapter 1 in this volume, Figure 1.5). Thus, making definitive mechanistic connections between the stressor and any secondary/tertiary measures is not always straightforward. Overall, however, our understanding of how the HPI axis and organismic performance varies throughout a stressful event (including recovery) is limited but is explored conceptually by Schreck and Tort (2016; Chapter 1 in this volume, Figures 1.5 and 1.6).

Studies linking the quantification of the stress response with fitness traits have the potential to provide insight into the physiological basis of life history. For example, relationships between stressor-induced plasma glucocorticoids and fitness (reviewed in Breuner et al., 2008) have been detected in both birds (eg, MacDougall-Shackleton et al., 2009) and reptiles (eg, Romero and Wikelski, 2001), with data emerging now in fish (Cook et al., 2014). This integrative approach of quantifying the stress response itself (or a component thereof) in combination with downstream changes in fitness facilitates collaboration between physiologists and ecologists, and advances interdisciplinary fields such as ecophysiology or conservation physiology (Wikelski and Cooke, 2006; Cooke et al., 2013; Boonstra, 2013a).

3. SPECIFIC MEASURES OF FISH STRESS

It is important to note that although quantification of stress is extensively studied and numerous indicators exist that identify a stressed fish (Tables 11.1–11.3), our grasp of what absolute levels of stress indicators mean is rudimentary, especially in nonexperimental contexts (eg, How do you know a fish in the wild is stressed or not?). Elevated levels of an indicator can signal a stressed fish, but lower levels of an indicator do not

Table 11.1
Cellular and molecular stress indicators

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Oxidative stress • Metabolic pathways produce reactive oxygen species (ROS) as a natural by-product (Costantini, 2008) • ROS are damaging to biological molecules, especially lipids, proteins, RNA, and DNA • Antioxidants prevent the damage caused by ROS either by preventing the formation of ROS, or by removing ROS • Oxidative stress occurs when ROS production overwhelms the counterbalancing capacities of antioxidants, and damage occurs to biological molecules (Lesser, 2006)	• Since oxidative stress can occur from either the overproduction of ROS, or from insufficient antioxidants, oxidative stress can be quantified by (1) measuring ROS; (2) measuring antioxidant levels; or (3) measuring damage to biomolecules • There are multiple markers available for measuring ROS, antioxidants, and biomolecule damage (Lesser, 2006) • ROS tend to be unstable, and so measurement of antioxidants or biomolecule damage are more common • Markers are measured through a variety of colorimetric assays depending on what is being measured, and different markers can be measured more easily in plasma, serum, urine, tissue homogenates, or cell cultures (Valavandis et al., 2006)	• Oxidative stress is an inevitable by-product of metabolism, and can therefore be taken as a cost of life • Oxidative stress has been measured in an ecological context as a cost of reproduction (eg, Alonso-Alverz et al., 2004), a cost of immune responses (eg, Torres and Velando, 2007), or a cost of strenuous energy expenditure such as migration (eg, Rankin and Burchsted, 1992) • Oxidative stress is also the result of exposure to challenging environments, such as areas that are heavily contaminated (eg, Bacanskas et al., 2004)	• Since oxidative stress arises from complex processes, results can be difficult to interpret • Measuring oxidative stress typically requires specialized equipment and can be relatively expensive, although commercial kits are becoming more widely available

Telomere length • Environmental stressors can cause oxidative stress, which if not counteracted can cause telomere shortening, accelerating cellular (and possibly organismal) senescence (Monaghan et al., 2009) • Environmental stressors such as psychological stress (Epel et al., 2004) or elevated reproductive effort (Kotrschal et al., 2007) have been linked to telomere shortening	• To determine relative telomere length, quantitative PCR can be used to measure the factor by which the DNA sample differed from a reference DNA sample in its ratio of telomere repeat copy number to single copy gene copy number (Cawthon, 2002)	• Potentially powerful indicator to bridge the gap between environmental stressors, oxidative stress, and organismal senescence	• The links between environmental stressors, cellular stress, telomere shortening, and organismal senescence are still largely untested (Monaghan and Haussmann, 2006). More research is needed to understand the tertiary outcomes associated with telomere shortening in order to effectively use telomere length as an indicator of stress
Heat shock proteins (HSPs) • HSPs, under the control of Heat Shock Factor 1 (HSF1), indicate a cellular stress response and HSP expression increases to maintain cellular homeostasis (Iwama et al., 2004) • Most HSPs are molecular chaperones which fold, repair, and catabolize proteins (Moseley, 1997)	• HSP expression can be determined using quantitative real-time PCR (qRT-PCR), requiring isolation of genomic DNA, total RNA extraction, and reverse transcriptase PCR amplification (eg, Fangue et al., 2006) • Hsp70 has previously been quantified by ELISA, BIAcore, and bead-based assays for use by FACS; BIAcore and FACS are more sensitive and require less sample than ELISA • HSP expression levels are context-dependent, meaning that the establishment of baseline expression levels is required (Tomanek and Somero, 1999)	• HSPs are sensitive to a range of stressors (eg, rapid temperature changes, salinity challenges, handling; Palmisano et al., 2000; Donaldson et al., 2008) • Widely studied, well-understood function	• The expression of HSPs is context-dependent since they are sensitive to the magnitude and duration of the stressor (Iwama et al., 2004) as well as acclimation to previous stressors (Somero and Hoffman, 1996)

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Table 11.1 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Immediate early genes and transcription factors • Immediate early genes (IEGs) are induced within minutes of the cellular stress response (Hughes and Dragunow, 1995) • Commonly measured IEGs include transcription factors c-fos, fosB, c-jun, JUNB, c-myc, egr-1 (Inuzuka et al., 1999) • Transcription factors, such as hypoxia-inducible factor 1 (HIF-1a) and NUPR1 are commonly activated during stress (Semenza, 1998; Momoda et al., 2007)	• IEG expression can be determined using qRT-PCR or microarray approaches • Can be measured in multiple tissues (eg, heart, liver, gill) • Nonlethal gill biopsies can be used, which facilitate integrative studies (eg, physiological telemetry; Miller et al., 2011)	• Sensitive indicators of stress and recovery (Momoda et al., 2007; Donaldson et al., 2014)	• Most studies have focused on mammals but interpreting IEG activation across a broader range of animal taxa would help identify upstream regulators of transcriptional stress responses (Kassahn et al., 2009) • Studies conducted on fish to date often focus on different timecourses (Krasnov et al., 2005), species, tissues (Kassahn et al., 2009), techniques (Prunet et al., 2008), and genes of interest • Future studies required to understand functional roles and downstream effects across species
Intracellular enzymes • Intracellular enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), or creatine kinase (CK), are released by cell damage or death • The presence of these enzymes in the plasma is therefore a useful indicator that tissue damage has occurred (Henry, 1996) • Many enzymes are tissue specific, and can therefore provide information about the type of tissue damage that has occurred (Wagner and Congleton, 2004)	• Indicators of tissue damage can be measured through colorimetric assays in plasma • Commercial labs also routinely measure enzymes in plasma using autoanalyzers for a fee	• The presence of intracellular enzymes in the plasma is a good indicator of injury • The availability of commercial labs makes measuring indicators of tissue damage relatively inexpensive and easy to measure in plasma without investing in specialized equipment	• These indicators are often not elevated unless physical injury has occurred, and so are less useful as an indicator of stressors that do not include tissue damage (Wagner and Congleton, 2004)

Table 11.2
Primary and secondary physiological stress indicators

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Catecholamines • When an individual is faced with a challenge, the physiological response is first an immediate release of the catecholamine hormones epinephrine and norepinephrine from the chromaffin cells (Reid et al., 1998; Gallo and Civinini, 2003) • The release of epinephrine and norepinephrine is associated with the classic fight-or-flight stress response	• Catecholamines are typically measured in plasma using chromatography with electrochemical detection (Woodward, 1982)	• Catecholamines are responsive to a variety of stressors (Reid et al., 1998; Pottinger, 2008), and the measurement of catecholamines therefore provides very accurate information about the response to acute stressors at fine timescales	• Catecholamines respond extremely rapidly (ie, within seconds) to capture and handling, and so it is difficult to quantify catecholamine levels without specialized equipment and animals that are held in the laboratory
Steroid hormones • Following the release of catecholamines, the stress response is characterized by activation of the hypothalamic–pituitary–interrenal (HPI) axis. HPI axis activation involves a complex set of interactions that culminate in the production and release of glucocorticoid steroid hormones (Mommsen et al., 1999; Barton, 2002; Pottinger, 2008)	• Steroid hormones are typically measured by either radio-immunoassay or enzyme-linked immunoassay in plasma or tissue homogenates (Pottinger, 2008; Sheriff et al., 2011) • Glucocorticoids can also be measured in urine and feces, and so can be extracted and measured in water samples (Ellis et al., 2004; Pottinger, 2008)	• Poststress glucocorticoid levels can provide information about how individuals are affected by specific stimuli (eg, capture and handling stress, different holding conditions, acute exposures; Sapolsky et al., 2000; Barton, 2002) • Baseline glucocorticoid levels can provide information about whether animals are experiencing chronic	• Circulating glucocorticoids respond rapidly (ie, often within 3–5 min) to capture and handling (Romero and Reed, 2005), and so it is often difficult to obtain baseline levels in wild animals • The relationships between both baseline (Bonier et al., 2009) and stress-induced (Breuner et al., 2008) glucocorticoid levels and future performance and survival

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Table 11.2 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
• Measurements of circulating glucocorticoids are therefore an indicator of whether an individual is experiencing a stressor • Corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH), the intermediate hormones involved in HPI axis activation, are also commonly measured	• Since glucocorticoids change rapidly to challenges, glucocorticoid measurements are often taken both before and after exposure to stressors to obtain a measure of stress responsiveness; both baseline and poststress levels, or stress responsiveness, provide information about the state of an individual (Breuner et al., 2008; Bonier et al., 2009)	environmental stressors, and in some cases can be predictive of future performance and survival (Bonier et al., 2009) • Stress responsiveness in some cases can be predictive of future performance and survival (Breuner et al., 2008) • Circulating glucocorticoid levels can also be linked to life history traits and trade-offs (Wingfield et al., 1998; Ricklefs and Wikelski, 2002)	are context- and species-specific, and results can be difficult to interpret
Metabolites • Once glucocorticoids are produced and circulating, they are associated with a suite of secondary responses that help the animal survive and recover from the challenge (Sapolsky et al., 2000), including the mobilization of stored glucose (Barton, 2002)	• Glucose and lactate can be measured through colorimetric assays in plasma or tissue homogenates • Lactate and glucose can also can be measured in whole blood using portable meters designed for diabetic patients or for athletic training (Wells and	• Metabolites are very useful for assessing the acute response to specific stressors (Barton et al., 2002), particularly exercise stressors in the case of lactate (Wood et al., 1983) • The readily available portable meters make both lactate and glucose inexpensive and easy to measure, using very	• Since both glucose and lactate are affected by general metabolic processes outside of stress responses, baseline results can be difficult to interpret, and these indicators are most useful as measures of acute responses to specific stressors

• Anaerobic stressors (eg, exhaustive exercise) generate anaerobic metabolites, such as lactate (Wood et al., 1983)	Pankhurst, 1999; Beecham et al., 2006; Stoot et al., 2014)	small blood samples with no specialized equipment, which makes them good parameters to measure under field conditions when assessing acute stressors (Wells and Pankhurst, 1999; Beecham et al., 2006)	
Osmolality and ion concentrations			
• When fish experience an acute stressor, the rise in adrenaline causes vasoconstriction and increased cardiac output (Mazeaud and Mazeaud, 1981), which in turn increases gill diffusing capacity due to increased perfusion of the lamallae (Randall and Perry, 1992) • This increased capacity for diffusion causes an increase in ion transfer at the gills, and subsequent changes in plasma osmolality, particularly in circulating concentrations of Na^+ and Cl^- (see McDonald and Milligan, 1997, for review)	• Plasma osmolality is measured using an osmometer • Ions can be measured in plasma using spectrophotometry • There are commercially available meters that will measure some of the common ions in plasma (eg, Na^+) • Commercial labs also routinely measure ions in plasma using autoanalyzers for a fee	• Changes in overall osmolality or ion balance are good indicators of acute stress • The availability of commercial labs makes measuring ions relatively inexpensive and easy to measure in plasma without investing in specialized equipment	• These indicators are useful indicators of acute stress, but are often difficult to interpret in the context of chronic exposures because they are context-specific and influenced by multiple internal and external factors (McDonald and Milligan, 1997)

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Table 11.2 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Nutritional indicators			
• Nutritional indicators in the plasma (eg, total protein, total cholesterol, triglycerides) provide information about current levels of mobilized energy stores that are available to fuel activities (Wagner and Congleton, 2004; Congleton and Wagner, 2006)	• Nutritional indicators in the plasma are typically measured through colorimetric assays • Commercial labs also routinely measure nutritional indicators in plasma using autoanalyzers for a fee	• Nutritional indicators in the plasma can provide information about the recent feeding history of fishes (Congleton and Wagner, 2006) • The availability of commercial labs makes measuring nutritional indicators relatively inexpensive and easy to measure in plasma without investing in specialized equipment	• Nutritional indicators do not show consistent responses to fasting and stressors, and results are species- and context-specific. Nutritional indicators can in some cases be difficult to interpret as indicators of general health (Wagner and Congleton, 2004; Congleton and Wagner, 2006; O'Connor et al., 2011)
Bioenergetics			
• Energy is the currency of life, so understanding its allocation to bodily processes can serve as a sensitive indicator to organismal stress (Beyers et al., 2002) • Energy stores and lipid content are linked to survival, reproduction, and life history strategies (Henderson and Tocher, 1987; Adams, 1999)	• Glycogen, a long-term energy reserve, is typically measured in tissue homogenates using hydrolysis and enzymatic assays • Phosphocreatine (PCr) and adenosine triphosphate (ATP) from tissue homogenates are typically measured through colorimetric assays • Proximate body composition (ie, proportion of the body that is lipid, protein, water, organic ash) and analysis can be performed to determine how energy is allocated among various compartments	• The availability of commercial labs makes measuring nutritional indicators relatively inexpensive and easy to measure in plasma without investing in specialized equipment • Provides long-term indication of organismal status • Information can be incorporated into bioenergetics models (particularly when combined with information on metabolic rates) to put in a broader context (Beyers et al., 2002)	• Since some indicators of energetic stores change very rapidly in response to acute exercise stress (eg, PCr or ATP); it is important to know the recent history of the animal in order to interpret results • Some measures such as proximate body composition analysis require relatively large quantities of tissue so tend to be lethal • Energy varies inherently among fish of different sex and size so need to control for these factors

	• Ω bomb calorimetry provides information on overall tissue energy density • Can also assess lipid constituents (eg, cholesterol, fatty acids, triglycerides), although these are rarely used as stress indicators • Commercial kits are available for measuring energetic stores in tissue homogenates	• Can use nonlethal electronic devices (eg, handheld microwave energy meter, Crossin and Hinch, 2005; bioelectrical impedance analysis, Kushner, 1992)	• Nonlethal sampling tools require calibration • Generally unresponsive to acute stressors (Schreck, 2000)
Leukocytes • Leukocytes, or white blood cells (WBCs), are a collection of cells in the blood that serve an important role in immune defense and inflammation • There are 5 types of WBCs in most vertebrates: basophils, eosinophils, lymphocytes, monocytes, and neutrophils (heterophils in birds and reptiles) • The relative proportions of each WBC type are influenced by stressors (Dhabhar et al., 1996), and thus provide a useful measure of animal health and exposure to stress	• Leukocyte profiles are typically obtained by light microscope examination of 100 leukocytes in a stained blood smear (Davis et al., 2008)	• Leukocyte profiles are predictive of future performance and viability, such as susceptibility to infection (Al-Murrani et al., 2002), growth rates (Moreno et al., 2002), and survival (Lobato et al., 2005; Kilgas et al., 2006) • Blood smears are relatively inexpensive and easy to obtain from captured wild animals • Leukocytes respond relatively slowly to capture and handling (ie, within hours or days; Davis et al., 2008), and so leukocyte profiles provide a convenient measure of baseline stress levels in wild animals	• Leukocyte profiles are influenced by disease and infection, as well as stress, and so it can be difficult to interpret changes in leukocyte profiles (Davis et al., 2008)

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Table 11.2 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
• The most common measure is the ratio of neutrophils or heterophils to lymphocytes (N:L or H:L ratio) (Davis et al., 2008)		• The leukocyte response is conserved across taxonomic groups, and so results obtained from one taxa should be widely applicable (Davis et al., 2008)	
Hematocrit • Erythrocytes, or red blood cells (RBCs), are the oxygen-transporting cells within the blood • Hematocrit is the volume percentage of RBCs in the blood	• Hematocrit is most commonly measured by packed cell volume (PCV), which is obtained by centrifuging a whole blood sample within a capillary tube, which separates the blood into layers • The volume of packed red blood cells divided by the total volume of the blood sample gives the PCV	• Hematocrit typically increases with exposure to stressors, is relatively inexpensive and simple to measure, and requires no specialized assays	• Hematocrit can in some cases increase or decrease in response to challenges, depending on the specific challenge, and results can be difficult to interpret

Table 11.3
Whole-organism stress indicators

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Reflexes • Simple reflex indicators such as ability to flip upright • Becoming increasing popular to characterize neurological responses of fish to external stimuli or functions of the autonomic nervous system (Davis, 2010)	• Reflexes can be assessed individually (as present or absent) or as a composite to derive a score (Davis, 2010) • Need to validate reflexes for each species but some common ones relevant to most species are righting-reflex (ie, roll fish on back and see if it regains upright orientation after 3 s) and tail-grab reflex (ie, grab the tail and see if fish attempts to burst away) • Does not require any specialized equipment and provides an immediate (<20 s) measure of fish vitality	• Predictive of mortality in a number of fish species in the lab (Davis, 2010) and field (Raby et al., 2012) • Rapid, simple, and inexpensive to evaluate reflexes without observer bias • Can train stakeholders (eg, anglers, fisheries observers), as it does not require any equipment or scientific skills • Developed given inability of traditional physiological measures to predict mortality (Davis et al., 2001) • Not dependent on fish size, motivation states, or acclimation (Davis, 2010)	• Need to validate reflexes for all species as not all fish have the same reflexes • Relatively new approach so relatively few published examples • Exact mechanisms by which reflexes predict mortality are unclear • Improper reflex choice or interpretation can be ambiguous

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Table 11.3 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Behavior • Locomotion, feeding, social interactions, predator-prey dynamics, habitat selection and other aspects of behavior • Behavior is an ecologically relevant indicator that requires integration of various physiological systems (Schreck et al., 1997)	• Can be measured in the lab and field • Growing number of tools for remotely studying the behavior of free-swimming fish using biotelemetry and biologgers (eg, electromyogram [EMG] telemetry; Cooke et al., 2004) • Action cameras provide opportunity to study fish behavior in water (Struthers et al., 2015)	• Ability to integrate with other measures including those more directly linked to organismal physiology (Scott and Sloman, 2004) given that physiology and behavior are inherently linked (Cooke et al., 2014) • Ecologically relevant given that many behaviors related directly to food acquisition and predator avoidance (Schreck et al., 1997) • Many behavioral endpoints are inexpensive (ie, no assay costs) but require specialized equipment that can be expensive or require technical expertise	• Difficult to identify specific mechanisms as behavior depends on capacity, motivation, sensory acuity, and responsiveness (Schreck et al., 1997) • Necessary to control for experimental artifacts and observer influence • Can be subjective (rather than objective) if endpoints are not clear
Swimming performance • Examples include speed, intensity, duration of swimming • Swimming requires the integration of numerous biological systems and is thus regarded as a sensitive integrator of stress and whole-organism status (Hammer, 1995)	• Most often quantified in laboratory environments with the use of swim tunnel/flume/annular respirometer (Ellerby and Herskin, 2013) or drag-strip (Nelson et al., 2002), although some swim tunnels are mobile	• Can be combined with other indicators such as metabolic rate (oxygen consumption) if swimming conducted in a respirometer (Farrell et al., 2003) • Swimming performance considered to have strong	• To some extent swimming performance is a reflection of fish motivation (eg, maturation, appropriate environmental cues), which can be independent of stressed state

	and can be used in field settings (Farrell et al., 2003) • Multiple forms of swimming (eg, burst, critical swimming speed, endurance) can be measured (see Beamish, 1978) • Repeat swimming performance approaches account for interindividual variation and are useful for evaluating performance impairments from stressors (Jain et al., 1998)	ecological relevance (Plaut, 2001)	• Initial costs associated with purchasing equipment can be relatively high • Swimming performance can be influenced by energetic state (fed vs fasted) (Gingerich et al., 2010)
Metabolic rate • Rate of oxygen consumption as food converted to energy • Indicates the minimum metabolic rate required to maintain life • There is likely a context-dependent link between metabolic rate with fitness, growth, or survival (Burton et al., 2011)	• Samples typically collected on resting, postabsorbtive animals, free of stimuli, in isolation, in a laboratory setting (Nelson and Chabot, 2011) • Can define standard metabolic rate, maximum metabolic rate, or aerobic scope (difference between maximum and standard metabolic rate)	• Elevation in metabolic rate can be interpreted as stressed state relative to controls (Barton and Schreck, 1987) • Can incorporate data into bioenergetics models to make inferences about several different processes	• Requires specialized equipment and standardized procedures to generate data (ie, animal must be postabsorbtive, isolated from external stimuli) • Factors such as handling stress, individual variation, social status, acclimation time, and nutritional status can all influence results (Sloman et al., 2000; Nelson and Chabot, 2011) • Data are strongly influenced by size, making comparisons across size classes challenging • Techniques are highly variable across research groups, making intraspecific comparisons challenging (Nelson and Chabot, 2011)

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Table 11.3 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Ventilation rate • Most fish move water over their gills through active ventilation, which involves opening and closing of the opercula, and can be used as a proxy for respiration (Barreto and Volpato, 2004)	• Can be counted with a stopwatch from direct observation or video (White et al., 2008) • Can be measured remotely by use of bioelectric sensors placed in water near fish (Altimiras and Larsen, 2000) • Possible to telemeter opercular activity (either radio or acoustic) to estimate ventilation rate (Oswald, 1978)	• Ability to differentiate between different stressors (Barreto and Volpato, 2006) • Relatively simple indicator that can be measured noninvasively and with little expense	• Ventilation rate does not appear to reflect the severity of a stressor in all species (Barreto and Volpato, 2004) • Rate alone might not be sufficient, and may be necessary to also quantify amplitude
Cardiac activity • The heart is essential for circulation and life-support such that cardiac activity (eg, heart rate, stroke volume, cardiac output) are relevant indicators of whole-organism stress (Farrell, 1991)	• Heart rate/ECG is relatively simple to measure, but, given that some fish are volume modulators, measuring cardiac output is often better (Farrell, 1991) • Doppler and transsonic cuffs can be used to measure blood flow in ventral aorta (Farrell, 1991)	• Robust indicator of stress in many species • Used to document responses to different husbandry conditions (Rabben and Furevik, 1993), environmental conditions (Claireaux et al., 1995; Lefrançois et al., 1998), and human-induced disturbances (Anderson et al., 1998)	• Baseline values of cardiac activity can be variable, making it difficult to identify when fish have recovered following a stressor • Although a large number of tools are available for measuring cardiac activity, they are all rather technically challenging

	• Heart rate can be measured remotely in free-swimming fish in the field (Priede, 1983; Armstrong, 1998) or in the lab using ECG biologgers (Raby et al., 2015) or bioelectric sensors placed in water near the fish (Altimiras and Larsen, 2000)	• Tend to be very responsive (eg, rapid change in the face of a stressor)	
Growth and Life History			
• Growth and reproduction occur only after the energetic demands of other processes are met • Reduced growth rate can result from stress • Several hard structures in fish (eg, scales, otoliths, bone) deposit growth rings to allow accurate age determination	• Can use a range of hard structures, some of which can be collected nonlethally (eg, scales) • Can generate proxy for reproduction with various gonadal indices	• Individual size correlates positively with fecundity in females, and with reproductive output in males of some species (Suski and Philipp, 2004) • Reduced growth rate is a well established indicator of stress (Pankhurst and Van der Kraak, 1997)	• May need lethal samples to generate data • May need to sample populations over long time scales to discern trends in growth, by which point it may be too late to stop or alter trajectories • Size/age alone might not be sufficient to discern population-level trends, and demographic data (ie, fecundity, survival) may be required • Need to validate growth across age classes (Beamish and McFarlane, 1983)
Condition indices			
• Condition indices of stress include length–weight relationships, organosomatic	• Approaches range from being relatively noninvasive (eg, simple measurements on live	• Simple and inexpensive (Bolger and Connolly, 1989) • Good indicator of the aggregate condition of the fish (eg, can	• Not overly sensitive to short-term stressors (Bolger and Connolly, 1989)

(*Continued*)

Table 11.3 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
indices, and necropsy (Barton et al., 2002)	fish) to lethal (eg, for organosomatic indices) • Organosomatic indices are ratios comparing the weight of an organ to body weight (eg, hepatosomatic index [liver:body weight, HSI], gonadosomatic index [gonads:body weight, GSI], viscerosomatic index [entire viscera:body weight, VSI], and splenosomatic index [spleen:body weight, SSI], Barton et al., 2002) • Values that are lower or higher than normal suggest that energy allocation to organs has been affected by stress (Kebus et al., 1992) • Necropsy-based methods (eg, health assessment index) requires autopsy of the sacrificed fish, whereby condition of internal organs is compared to published standards that outline criteria of an organ of normal condition (Adams et al., 1993)	detect chronic stress; Barton et al., 2002) • Some nonlethal options (eg, length–weight analysis, condition factor, relative weight)	• Some critiques regarding the use of condition indices given that they can lead to inappropriate conclusions based on inherent limitations of the various methods (Cone, 1989) • More involved measures (organosomatic and necropsy) are lethal • Condition indices can be influenced by seasons, stage of development, sexual maturation, and disease state • Often require large effect size to detect stress

Fluctuating asymmetry			
• Differential development of a structure on one side of an organism (Jagoe and Haines, 1985) • Symmetrical structures should result from the same genetic material, so deviations from symmetry can represent stress in the form of genetic mutation, or environmental stress (Leary and Allendorf, 1989)	• Quantifying the presence of fluctuating asymmetry can indicate stress within a population • Can be used as an early warning indicator prior to population-level declines in abundance or adverse environmental conditions (Jagoe and Haines, 1985)	• Can be inexpensive and straightforward to measure as meristic/morphometric characters are measured or counted	• Many meristic characters (eg, fin rays) are variable, making them unreliable as stress indicators (Leary and Allendorf, 1989) • Not all species or characters lend themselves to studies of fluctuating asymmetry as a stress index • May require large sample sizes to identify trends/patterns (Jagoe and Haines, 1985) • May be challenging to define a causal mechanism or link between asymmetry and stress (Jagoe and Haines, 1985)
Reproduction and fitness			
• Reproduction and reproductive output is a process critical to survival and persistence of an individual and a species • Reduced reproduction can result from stress	• Can assess extent of intersex as an indicator of environmental stress (Bortone and Davis, 1994) • Impacts of stress may be visible in a range of reproduction-related factors such as gamete quality and/or timing of reproduction (Schreck et al., 2001)	• Very ecologically relevant when relating stress to population-level parameters • Can measure a suite of gonadal indices (mass, size, egg stage, hormones, etc.) as proxies for reproduction • Can perform artificial crosses with known parents to define offspring survival and viability (Campbell et al., 1994)	• May need to collect data on reproduction only at certain times of the year • May be difficult to assign a cause-and-effect relationship between a stressor and reduced reproduction

(*Continued*)

Table 11.3 (Continued)

Indicator	Sampling and analytical considerations	Strengths of indicator	Weaknesses of indicator
Survival • The most extreme response to a stressor is death, whereby homeostasis cannot be maintained (Wood et al., 1983)	• Mortality can be measured by holding fish in nets, cages, pens, or tanks and simply counting the dead (Gutowsky et al., 2015) • Often instructive to mark/tag individual fish to determine which individuals died and their history • Increasing set of tools (eg, biotelemetry) available to study mortality in free-swimming fish in the wild (Pine et al., 2003)	• Often simple, inexpensive approach • Mortality is absolute and a clear, ecologically relevant fitness indicator • Most powerful when combined with other indicators that reveal the mechanistic basis for mortality (eg, Cooke et al., 2006)	• May be difficult to obtain animal care and use approvals to use death as an endpoint • Subject to bias from method used to assess mortality (eg, net or cage effects; Gutowsky et al., 2015) • Fish can die for many reasons (eg, senescence) such that it is necessary to have appropriate controls

necessarily equate to a less stressed or unstressed fish. Readers are encouraged to consider this caveat when investigating and selecting measures of fish stress.

3.1. Cellular and Molecular Indicators

Acting in concert with the responses that occur at higher levels of biological organization, the cellular and molecular suite of stress responses help to temporarily tolerate stressors (Kultz, 2005, Table 11.1). Cortisol is involved in molecular responses to stressors as it stimulates the expression of metallothionein, ubiquitin, and HSPs by interacting with heat shock factors (HSFs), which affect transcriptional regulation (Vamvakopoulos and Chrousos, 1994; Kassahn et al., 2009). Cortisol also binds with glucocorticoid receptors and interacts with the c-Jun component of the activation protein-1 (AP-1) transcription factor (Iwama et al., 2006). The transcriptional effects of cortisol binding to glucocorticoid receptors depend on tissue type and HSP 90 expression levels (Basu et al., 2001; Vijayan et al., 2003). A range of stressors induce a common set of responses, which can include repair of DNA and protein damage, cell cycle arrest or apoptosis, the removal of cellular and molecular debris, and changes in cellular metabolism that reflect the transition from anabolic to catabolic states (Iwama et al., 2004). Cumulatively, these cellular and molecular responses are triggered by the eukaryote minimal stress proteome, a set of evolutionarily conserved proteins (Kultz, 2005) (Faught et al., 2016; Chapter 4 in this volume).

Stressor exposure results in the production of reactive oxygen species (ROS), resulting in oxidative stress inside the cell. Oxidative stress leads to increased levels of protein damage. The amount of ubiquitin-labeled protein can indicate the level of protein damage in the cell (Iwama et al., 1998; intracellular enzymes also indicate cell damage or death, see Table 11.1). The damaged and ubiquitinated protein in the cell induces a heat shock response, aimed at repairing protein damage (Wu, 1995). The magnitude of the heat shock response depends on the magnitude and duration of the stressor and acclimation state (Iwama et al., 2004; Somero and Hofmann, 1996). HSP expression is regulated by heat shock factor 1 (HSF1), which dissociates HSPs from HSF1 following activation of the hypothalamic–pituitary–interrenal (HPI) axis. HSF1 then migrates to the nucleus and begins the transcription of HSPs (Kassahn et al., 2009). ROS can also accelerate the reduction in telomere length resulting from oxidative stress. Telomeres are the caps at the end of the chromosome that are essential for genome stability, and, when reduced at an accelerated rate can, in turn, hasten cell senescence (Richter and von Zglinicki, 2007). The concept that telomere length can be correlated with cellular senescence, and possibly

organismal senescence, has become an emerging field of research (Ricklefs, 2008; Monaghan et al., 2009). A range of environmental stressors, including psychological stress (Epel et al., 2004) or elevated reproductive effort (Kotrschal et al., 2007), can affect telomere length in vertebrates. So there is evidence that environmental stressors can cause oxidative stress, which if sustained, may trigger cellular senescence and ultimately organismal senescence (Monaghan et al., 2009).

Changes in gene expression (ie, quantitative, qualitative, and changes in reaction coefficients) can be linked with a range of stressors (Krasnov et al., 2005). Genomics tools such as microarrays and gene expression profiling are now commonly used to understand the responses of fish to a range of stressors. Commonly examined stressors include temperature (eg, Jeffries et al., 2012), hypoxia (eg, Gracey et al., 2001), handling stress (eg, Donaldson et al., 2014), and toxicants (Williams et al., 2003). cDNA microarrays enable thousands of genes to be screened simultaneously to identify groups of differentially expressed genes related to biochemical pathways involved in a range of responses. Relatively few studies have assessed gene expression in relation to acute stressor exposure, compared to the literature assessing primary, secondary, and tertiary stress indicators (Caipang et al., 2008; Prunet et al., 2008). Instead, one of the main objectives of functional genomics studies is broadening our understanding of how gene expression is influenced by environmental conditions (Buckley, 2007; Miller et al., 2009). Microarrays using nonlethal tissue biopsies (ie, muscle and gill tissue) have identified potential genes involved in an unhealthy signature of migrating sockeye salmon, where individuals that are less likely to reach spawning grounds are characterized by indices of poor health, including downregulation of blood clotting factors and genes related to aerobic respiration, as well as expression of genes linked with immune function (Miller et al., 2011). Still, understanding the functional significance of many genes and gene families remains a challenge, and the implications of how genes respond following exposure to a stressor is not always clear.

In fish, a number of genes have been investigated as potential biomarkers for various stressors. Genes linked to cell apoptosis, such as cytochrome c and transcription factor JUNB, are upregulated in response to elevated temperatures in sockeye salmon (*Oncorhynchus nerka*; Jefferies et al., 2012). JUNB is likewise upregulated following low-water and air exposure stressors in rainbow trout (*O. mykiss*; Momoda et al., 2007). Transcription factor NUPR1, which is involved in the regulation of cell growth and apoptosis (Mallo et al., 1997), is also responsive to stressors in rainbow trout, and can remain upregulated several hours poststressor (Momoda et al., 2007). Changes in the gene expression of biological pathways related to inflammation, protein degradation, and the immune response have been

observed in rainbow trout (*O. mykiss*) liver in relation to handling stress (Momoda et al., 2007; Wiseman et al., 2007). Also in rainbow trout, following repeated exposure to a netting stressor, Krasnov et al. (2005) observed changes in expression of genes related to immune responses, cell proliferation and growth, apoptosis and protein biosynthesis in the brain, and changes in expression of genes related to cellular biochemical processes in the kidney. The onset of recovery poststress may be evidenced by the expression of genes related to gluconeogenesis, glycogenolysis, and energy metabolism in the liver (Momoda et al., 2007; Wiseman et al., 2007). Donaldson et al. (2014) identified species- and sex-specific genomic responses in sockeye salmon related to the stress response and recovery following exposure to an exercise stressor. A complicating factor in understanding the behavior of these genes during the stress response is the fact that studies conducted on fish to date often focus on different time courses (Krasnov et al., 2005), species, tissues (Kassahn et al., 2009), techniques (Prunet et al., 2008), and genes of interest.

3.2. Primary and Secondary Physiological Indicators

Physiological indicators of stress include all the responses between the cellular and molecular level and the whole-animal level (Table 11.2). The stress response involves a wide range of physiological responses including, and beyond, HPI axis activation (Schreck and Tort, 2016; Chapter 1 in this volume, Figure 1.6). There are a suite of primary indicators (eg, catecholamines and stress hormones), as well as secondary responses (eg, changes in glucose, ion balance, acid–base balance, immunological functions, or other indicators of energetic metabolism) that can be used to assess stress in fish. Secondary changes happen over a slower timescale than the primary responses. The use of the different primary and secondary stress indicators has relative advantages or disadvantages depending on the stressor of interest, and the level of background information available about the species, population, or individual (Table 11.2).

Changes in certain primary and secondary stress indicators are notably useful when assessing responses to specific aquaculture or handling practices, or acute disturbances in the field. Catecholamines provide the fastest primary response to stressors, but are difficult to measure because they respond quickly to stressor exposure (Reid et al., 1998; Pottinger, 2008). Catecholamines are appropriate and powerful indicators in laboratory settings, but are often not logistically possible to measure in the field because they are highly responsive to capture and handling. Cortisol, the other measure of the primary stress response, is among the most commonly measured stress indicators (Mommsen et al., 1999; Barton, 2002; Pottinger,

2008). Because cortisol responds more slowly than catecholamines to specific stressors, it can be quantified in laboratory or field settings to obtain both baseline and poststressor levels, as long as animals can be sampled within a few minutes of capture (Romero and Reed, 2005). Secondary stress indicators commonly measured include (1) glucose elevation, a result of the increase in catabolism and release of glucose into the blood stream following stressor exposure (Barton, 2002); (2) lactate elevation, an indicator of anaerobic metabolism following hypoxia or exercise stressors (Wood et al., 1983); (3) osmolality or specific ions, which are altered as a result of the increase in catecholamines and subsequent increase in heart rate and gill permeability (McDonald and Milligan, 1997); and (4) leukocytes, which can not only reflect response to acute stressors, but may also predict future survival or performance (Davis et al., 2008).

Many of these physiological indicators are measured in plasma as they provide a snapshot of the circulating levels of the hormone or metabolite (Barton, 2002; Pottinger, 2008). The benefit of sampling plasma (or red blood cells for indicators such as hematocrit) is that, for most fishes, samples can be collected nonlethally. Also, because plasma is a conventional tissue for measuring physiological parameters, there are a number of studies that exist to aid in the interpretation of values, and assays or meters are commercially available to facilitate data generation (Wells and Pankhurst, 1999; Beecham et al., 2006). The main disadvantage of measuring indicators in plasma is that plasma is not always the most relevant tissue, and some indicators do not make sense for plasma measurements. For example, bioenergetics indicators measured following chronic stressor exposure such as glycogen liver stores could not obtained by taking only a plasma sample. Similarly, as phosphocreatine (PCr) and adenosine triphosphate (ATP) are indicators of acute exercise stress, these metrics are best measured in muscle tissue. Therefore, the specific tissue sampled will be dependent on the research question, and the primary/secondary physiological indicator of interest. Taking these caveats into account, primary and secondary stress indicators provide information about how individuals are perceiving and responding to environmental challenges, and can identify the extent to which animals are stressed by these challenges, as well as potentially predict future performance and survival (Breuner et al., 2008).

3.3. Whole-Organism Indicators

Whole-organism (or tertiary) responses to stressors include a number of aspects of fish performance such as changes in growth, condition, disease resistance, metabolism (Sadoul and Vijayan, 2016; Chapter 5 in this volume), cardiac activity, swimming performance, behavior, fitness, and even survival

(Wedemeyer et al., 1990, Table 11.3). These responses can serve as indicators of stress, and are also generally considered to have ecological relevance. For example, growth rates can directly influence population models (Power, 2002) and bioenergetics models (Beyers et al., 2002), while fitness and survival influence demographic processes (McCallum, 2000). Behaviors related to food acquisition, predator avoidance, and habitat selection also have direct ecological relevance with a physiological underpinning (Godin, 1997). Many condition-related measures involve simple measures of mass (ie, usually the entire organism along with specific organs), length, or both (Goede and Barton, 1990). Traditional measures of fish condition (eg, relative weight, condition factor) are also simple to calculate, but are criticized for their lack of specificity and breadth (Bolger and Connolly, 1989). So-called organosomatic indices (eg, ratio of organ mass to body mass) are widely used as an index of stress and condition given their simplicity, but they require lethal sampling. Some researchers have generated indices that are a composite of various measures related to body condition and health, and perhaps the most commonly used is the Health Assessment Index (HAI), first proposed by Adams et al. (1993). The HAI can be modified to meet the needs of researchers and their study questions.

Swimming performance represents another tertiary endpoint that is functionally simple to both collect and interpret, and is also ecologically meaningful, but proper measurement requires custom-designed swim tunnels or flumes that can be technically challenging to build, or expensive to purchase. Critical swimming speed is an example of a metric that has been measured for decades (Beamish, 1978), and although it is now regarded as somewhat limited in value (Plaut, 2001), it does have use when making relative comparisons of fish swimming ability among stressor treatments. Portz (2007) reported that if the goal of a study is to simply make treatment-level comparisons in swimming ability, then simply defining the time to exhaustion for fish chased by tail pinching in a round tank generated values that correlate with more formal measures of critical swimming speed. Recognizing inherent variation in swimming ability among individual fish, Jain et al. (1997) refined swimming protocols to create a new method that compared the ability of fish to swim a second time shortly after doing an initial swim. Termed the "recovery ratio," the approach has served as a sensitive indicator of overall organismal status in the face of different stressors (see Jain et al., 1998). When we combine measures of cardiac activity (eg, heart rate or cardiac output) and metabolic rate (oxygen consumption) with active swimming challenges (see Webber et al., 1998), it is possible to obtain a multilevel understanding of stress and its influence on an ecologically relevant index of organism performance. In fact, laboratory-derived relationships between metabolism, cardiac function, swimming ability, and water temperature can be scaled to field

environments to predict mortality (Farrell et al., 2008). Together, the measurement of fish swimming ability, using a range of possible techniques, has the ability to provide valuable information on how stress can influence an important aspect of fish ecology.

Tertiary stress indicators are more diverse than data on the primary or secondary stress response, which tend to fall almost exclusively within the realm of the physiologist. Collecting data related to the tertiary stress response, as well as interpreting those data, may require the expertise of ethologists, field ecologists, as well as physiologists. Nonetheless, there certainly are strong physiological underpinnings, some more direct than others, to tertiary stress indicators. Indicators related to cardiac activity and metabolism are the domain of physiologists, but also link to activities such as swimming (both swimming performance and general locomotor activity) and other elements of behavior. Metabolism and locomotor activity are large drivers of energy budgets (Boisclair and Leggett, 1989). Energy budgets define somatic growth rates (and condition), reproductive investment, and behavior. In that sense, many of the tertiary indicators are linked to bioenergetics (Beyers et al., 2002). Even the growing interest of using reflex impairment as indicators of fish vitality is linked to metabolism and neurological function (Davis, 2010).

As we move from measures focused on direct indicators of homeostasis (ie, primary or secondary stress indicators) to more whole-organism level indicators that represent integration of various mechanisms (eg, neuroendocrine processes), the ability to infer a stressed state becomes more challenging, and may be best studied in the context of comparisons among groups (eg, control vs stressor A and stressor B). For example, something as straightforward as mortality is a natural phenomenon, and a dead fish does not immediately imply that the fish was stressed; senescence is a natural process, and a fish can be predated upon independent of whether it was in a stressed state. Similarly, reduced feeding behavior of a fish does not indicate that the fish is experiencing stress because it may simply not be hungry, which could be influenced by previous feeding history, metabolic demands, food availability, seasonality, or even genetics. This is unlike primary and secondary stress indicators, such as cortisol or osmolality, for which there are clear reference ranges or where a specific threshold (eg, X ng/mL or X mmol/L) is meaningful. Also, circulating cortisol for an individual fish may be elevated relative to reference ranges, yet there are no organism-level endpoints evident. Does that mean that the fish is stressed? Context clearly matters, which means comparisons using appropriate controls must be used when inferring stress from tertiary endpoints.

Despite these challenges, the elegance of using tertiary indices to define stress is their simplicity, both in collection as well as interpretation, particularly in relation to other physiological measures of stress that can

only be quantified with laboratory work. For example, two metrics that are simple, elegant, as well as highly relevant when identifying whole-organism stress, are reflex indicators (eg, is a specific reflex present or absent) and survival (eg, did an organism live or die). What is remarkable with these metrics is that something as simple as reflex impairment has been shown to correlate with survival (Davis, 2010; Raby et al., 2012), and is now being widely embraced by the research community to define the physiological state of animals in the field, and to predict mortality. Interestingly, however, the mechanistic basis for reflex impairment is not directly clear. Similarly, many tertiary indices of stress can be measured outside the traditional laboratory with wild, free-swimming fish. For example, some measures such as fine-scale locomotor activity (Cooke et al., 2004), cardiac activity (Cooke et al., 2004; Clark et al., 2010), and plasma glucose (Endo et al., 2009) can be measured remotely and either transmitted (ie, biotelemetry/biosensors) or stored aboard electronic tags for later downloading (ie, biologging). Reflex impairment measures can also be conducted in field settings, as they do not require any specialized equipment (Raby et al., 2012).

4. CONSIDERATIONS FOR MEASURING AND INTERPRETING STRESS

With regard to the stressor, study animal, and stress indicator, there are a number of factors to consider when quantifying and interpreting stress in fishes (Fig. 11.3). A selection of these factors is highlighted next.

4.1. Interspecific Differences

It is perhaps not surprising that different species of fish respond to the same stressors differently. Given evolutionary, ecological (ie, predation), environmental (ie, temperature), and life history differences that contribute to the divergence of species, HPI activity and other indicators of stress are apt to vary. Comparing stress across species is largely limited by variation among studies in stressor type, severity, and duration, which can dramatically affect the magnitude of the HPI stress response (see Section 4.4). When exposed to an identical stressor, plasma cortisol (Barton, 2000, 2002; Pottinger, 2010) and glucose (Jentoft et al., 2005) levels, gene expression (Jeffries et al., 2014b), immune function (Cnaani et al., 2004), habitat preference (Jacobsen et al., 2014), and avoidance behavior (Hansen et al., 1999) all can vary among species, including closely related species (eg, pink salmon, *Oncorhynchus gorbuscha* and sockeye salmon, *O. nerka*;

Donaldson et al., 2014). However, under certain conditions, different species can exhibit similar responses to the same stressor. Campbell et al. (1994) noted that repeated air exposure reduces progeny survival to a similar degree in rainbow and brown trout (*Salmo trutta*). Ryer et al. (2004) showed that juvenile sablefish, *Anoplopoma fimbria*, known to be robust against fisheries capture mortality, display similar behavioral impairment following capture as the more fragile walleye pollock, *Theragra chalcogramma*. Design of experiments whereby multiple species (and where possible, hybrids; eg, Noga et al., 1994) are exposed to identical stressors, and identical indicators are measured, can maximize collection of data that aid in understanding factors driving species-specific differences in stress.

4.2. Intraspecific Differences

Measures of stress can also vary within species. Often the existence of unique populations motivates examination of stress. Environmental and ecological factors linked to geographically distinct populations are predicted to drive divergence in stress responsiveness. Indeed, along a longitudinal gradient, populations of killifish, *Fundulus heteroclitus* (northern vs southern) vary in their stressor-induced plasma cortisol responses (DeKoning et al., 2004). Stressor-induced changes in ventilation rate of tropical poeciliids, *Brachyrhaphis episcopi* (confinement stressor, Brown et al., 2005) and three-spined stickleback, *Gasterosteus aculeatus* (predator stressor, Bell et al., 2010) vary depending on whether fish are collected from low- or high-predation habitats. In other instances, differences in physiological parameters of stress (eg, plasma cortisol, lactate, glucose) are not present among populations but fitness outcomes (ie, survival) can still vary poststressor exposure (Donaldson et al., 2012). Population-specific differences in the stress response are intriguing from an evolutionary standpoint, and should not be overlooked when making specieswide conclusions on stressor sensitivity.

Other intraspecific factors driving variation in stress indicators include sex, size, social status, and domestication. Sex differences can be especially pronounced in certain species. Adult female Pacific salmon (*Oncorhynchus* spp.) have higher stressor-induced plasma cortisol levels (Donaldson et al., 2014) as well as a greater likelihood of mortality (Martins et al., 2012) in response to temperature stressors. Timing of peak stressor-induced plasma cortisol is affected by body size in European sea bass (*Dicentrarchus labrax*; Fatira et al., 2014). Poststressor HPI activity is known to vary between dominant and subordinate rainbow trout (Jeffrey et al., 2014a). Variation in measures of stress are also present between hatchery and wild trout (Woodward and Strange, 1987; Lepage et al., 2000) and salmon (Johnsson et al., 2001), which in combination with all intraspecific considerations has

implications with regard to maximizing performance of fishes reared for captivity or release into the wild for stock enhancement.

Finally, there is a rapidly developing appreciation for heritability of, and interindividual/interfamily differences in, stress-responsiveness (eg, Kittilsen et al., 2009; Pottinger, 2010; Hori et al., 2012), as well as coupling of physiological and behavioral stress indicators within an individual (ie, coping style, Castanheira et al., 2015). Aquaculture has utilized this variation for over a decade to breed genetically divergent lines of rainbow trout (*O. mykiss*) with high and low plasma cortisol responses to a 3 h confinement stressor (Pottinger and Carrick, 1999). This variability is now associated with consistent variability in myriad of behavioral indicators of stress (eg, activity following a conspecific intruder, Øverli et al., 2007). High and low responders have also been characterized in Atlantic salmon (*Salmo salar*, Fevolden et al., 1991), gilthead sea bream (*Sparus aurata*, Tort et al., 2001), Atlantic cod (*Gadus morhua*, Hori et al., 2012), and striped bass (*Morone saxatilis*, Wang et al., 2004). These individual-level differences may also influence individual tolerance to the sampling methodology to quantify stress (eg, recovery from cannulation, Bry and Zohar, 1980). Together, there are multiple aspects of intraspecific variation that can influence stress indicators; accounting for and describing this variation is warranted.

4.3. Context-Specific Differences

HPI axis activity, in addition to auxiliary molecular, physiological, and whole-animal responses can vary in magnitude depending on the environmental and ecological context of the stressor an individual is exposed to. Activation of the HPI axis should be consistent across contexts. Ultimately, any threat to an animal's fitness must be endured and overcome via HPI-mediated changes to physiology and behavior (Schreck and Tort, 2016; Chapter 1 in this volume). The changes to physiological and behavioral processes may nonetheless differ if the individual is faced with a predator, resource competition, restrictive feeding, hypoxia, aquatic pollution, or elevated water temperature.

Context-dependent variation in stress measures is observed at all organizational levels. At the neuroendocrine level (primary indicator), changes in brain monoamine (eg, norepinephrine, serotonin) concentrations differed in three-spined stickleback based on stressor type (unfamiliar conspecific vs predator; Bell et al., 2007). At later stages of the HPI axis, predator-induced whole-body cortisol response of winter flounder (*Pseudopleuronectes americanus*) differed depending on species of predator (Breves and Specker, 2005). More subtle differences in stressor type may not elicit different plasma responses (eg, intra- vs interspecific intruder, Ros et al.,

2014). Interestingly, two very different stressors with regard to ecological relevance, and potentially severity, can elicit similar responses. Visible implant elastomer tagging elicits a similar plasma cortisol response in three-spined stickleback as that following a simulated predator attack (Fürtbauer et al., 2015). Secondary, physiological processes are also influenced by stressor context. Degree of change in metabolism varied in *Galaxias maculatus* (Milano et al., 2010) depending on whether individuals were exposed to visual or olfactory predator cues. At the whole-animal level, change in ventilation rates varies among stressors (eg, confinement vs conspecific vs electroshock, Barreto and Volpato, 2006). Likely interlaced with stressor severity (see Section 4.4) the environmental and ecological context of a stressor matters when making decisions about which indicator to select, predicting the response of the indicator, and interpreting the results.

When assessing stress, exposure of animals to multiple stressors is arguably the most biologically relevant context. Experimental design incorporating different stressors (eg, elevated water temperature and fisheries capture, aquatic pollution, or immune challenge) may detect magnification of stress indicators (eg, Marcogliese et al., 2005; Jacobsen et al., 2014). Fish are also exposed to the same stressors more than once, and previous experience with a particular stressor and the individual's capacity to learn may influence stress indicators (Barcellos et al., 2010; also see discussion on habituation in Section 4.4). Housing conditions prior to stressor exposure (ie, isolation vs groups) can also influence stress indicators (Giacomini et al., 2015), potentially via communication of olfactory cues of conspecific stress (Barcellos et al., 2014a). Testing fish singly versus in groups can be motivated by the schooling and social tendencies of the species. Incorporating additional experimental variables to increase biological relevance can introduce other logistical challenges such as requirement of larger sample sizes. If the scientific priority is for biological relevance of stress indicators, such nuances of ecological and environmental context can be considered.

For some contexts, identifying suitable indicators is intuitive (eg, measuring ventilation frequency and avoidance behaviors following a simulated predator attack). Other scenarios allow for use of many suitable indicators. Exposure to toxicological stressors affects gene expression (Jeffries et al., 2015), gamete quality (Khan and Weis, 1987), and suites of physiological processes and behaviors across life stages (Scott and Sloman, 2004; Sloman and McNeil, 2012). Approaches using multiple indicators (eg, Woodley and Peterson, 2003) provide opportunities to discover or exclude indicators for a particular stressor. However, it is necessary to keep in mind that primary and secondary indicators of stress may not correlate with whole-animal or tertiary indicators (eg, HSP expression increasing in response to a thermal challenge but swimming

performance remaining constant). Even within a class of indicators, responses can vary (eg, increases in lactate concentration indicating insufficient oxygen supply to muscles, but no change in HSP expression indicating absence of protein damage). Accordingly, the nature of the stressor or stressors, and relationship among indicators, is important to consider when determining the appropriate indicators of stress to measure.

4.4. Stressor Severity

As discussed by Schreck and Tort (2016; Chapter 1 in this volume), embedded within context-specific differences is whether the stressor will be applied in an acute/single, repeated, or chronic/prolonged manner. An acute or single exposure may be typified by brief durations (seconds to minutes), and associated with physiological responses that are adaptive. Variation in duration of an acute stressor can alter stress indicator levels in a gradated manner (Gesto et al., 2013, 2015). Acute stressors can also vary in intensity with regard to expected level of impairment (eg, 2-minute confinement vs 5-minute chase with net), again resulting in variation in stress indicator levels (Geslin and Auperin, 2004). For contaminant stressors, there are often clear patterns between dose concentration and extent of indicator response or mortality (ie, LC_{50}). However, as mentioned in Section 4.3, stressors that may be considered distinct based on severity can elicit similar responses, as well as identical stressors differing in duration (Fatira et al., 2014).

Definitions of what constitutes a chronic stressor exposure are not always consistent; chronic stress can be repeated, sequential exposure to an acute stressor (eg, daily handling over multiple weeks), or continuous prolonged exposure to a stressor (eg, continuous exposure to elevated water temperature over multiple weeks). How an indicator responds to these exposures may depend on its role in immediate (eg, avoidance behavior) versus long-term (eg, growth) effects on fitness (Schreck, 2000). Also, for some species, chronic stressor exposure is reality (as in Boonstra, 2013b). Although indicators may imply a chronically stressed state (ie, allostatic overload, McEwen and Wingfield, 2003), the responses may be adaptive (Boonstra, 2013b). For repeated exposures, the interval between stressor application can affect the response of the stress indicator as well (Schreck, 2000). Thus, habituation must be considered in order to validate that changes to stress indicators are indeed due to "disrupted negative feedback" (Romero et al., 2009) or allostatic overload (Schreck and Tort, 2016; Chapter 1 in this volume, Figures 1.3, 1.5, and 1.6).

Discerning habituation from desensitization and exhaustion using hormonal indices of stress is discussed by Schreck (2000) and Cyr and Romero (2009). Defining habituation using performance/behavioral

indicators may not be as straightforward and measurement of an accompanying physiological parameter (ie, HPI axis activity) is needed. A subset of animals can be sampled midway through repeated exposure to an acute stressor (eg, immediately following daily handling stressor being applied over multiple weeks) to confirm that the HPI axis and stress indicator is still responsive to the stressor. A subset of animals can be exposed to an acute stressor midway through a sustained stressor exposure (eg, single, acute chase stressor during continuous exposure to elevated water temperature over multiple weeks) to confirm that the HPI axis and target stress indicator is still responsive. Diminution of the endocrine stress response is detected in a number of species chronically stressed (Barton et al., 1987; Jentoft et al., 2005, but see Barcellos et al., 2006). Wingfield et al. (2011) proposed that there are thresholds after which mounting a stress response is no longer adaptive. When these thresholds are surpassed, resistance potential to the stressor increases via attenuation of the stress response (Wingfield et al., 2011). Multiple samplings throughout the chronic exposure may identify tipping points whereby the stress indicator no longer responds (resilience) or recovers to baseline, prestressor levels (exhaustion).

Phenotypic plasticity should also be considered, particularly when quantifying stress in wild populations inhabiting environments with fluctuating conditions that are also chronic stressors (eg, climate change, aquatic pollution, Silvestre et al., 2012; Crozier and Hutchings, 2014). Laboratory-based indicators of stress may not be reliable indicators in wild animals (Dickens and Romero, 2013), and this may be due to shifting coping strategies. Alternatively, laboratory-based indicators of chronic stress may be detected in wild populations but without population-level consequences. Populations of invasive round goby from highly contaminated areas in Lake Ontario demonstrate indices of toxicological stress (eg, endocrine disruption, Marentette et al., 2010; impaired behaviors, Sopinka et al., 2010). Yet, populations of round goby in polluted areas are stable and populations in reference areas are declining (McCallum et al., 2014). This example illustrates how new organismal steady states may emerge and be interpreted as stress, but do not affect population-level processes (see Section 5).

4.5. Field Versus Laboratory

Laboratory and field measurements of stress each have their advantages and disadvantages. Certain indicators of stress must be measured in the laboratory due to the complexity of equipment used to obtain samples (eg, serial blood sampling via cannulations, Fig. 11.1). Other indicators can only be measured in the field due to the inability to replicate the behavior in the laboratory (eg, stressor-induced changes in migration rates, Donaldson

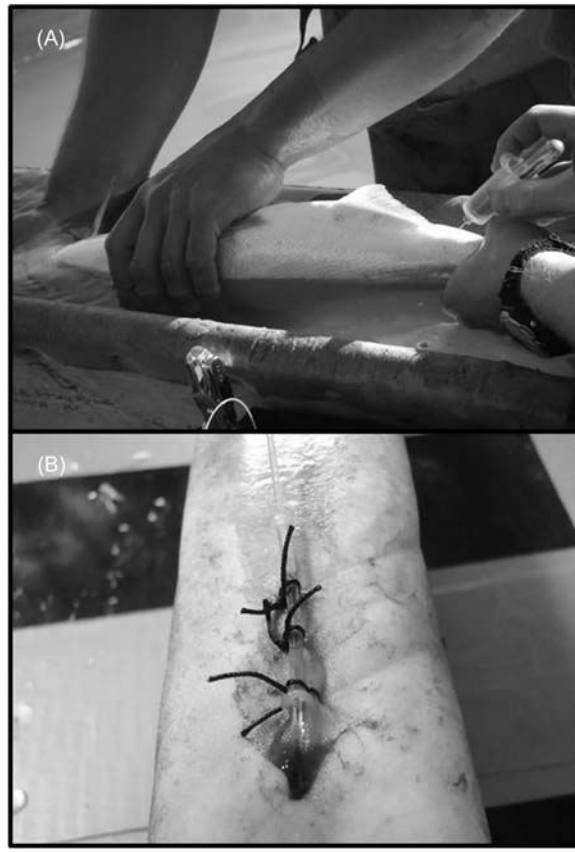

Figure 11.1. Blood can be collected from live fish to measure stress indicators by (A) drawing blood from the caudal vasculature using a syringe or Vacutainer; or (B) by implanting a cannula in the vasculature (often aortic), which also enables serial sampling. Photo credits: (A) Michael Donaldson, (B) Michael Lawrence.

et al., 2011). Laboratory studies allow for control of variables that can confound measurement of stress indicators, and otherwise are difficult to control in the wild (eg, water temperature). This degree of control is crucial and necessary for research targeting mechanistic connections between stressor and indicator. Still, mimicking stressors that fish will encounter in the wild in a laboratory setting does not truly encompass the entire stress response that an animal would elicit under ecologically relevant conditions. For example, latent effects of sublethal stress indicators (eg, postrelease predation following a fisheries capture stressor that impairs equilibrium,

Danylchuk et al., 2007) are not adequately accounted for in the laboratory. Depending on study hypothesis and goals, measurement of the chosen stress indicator may be more suitable under laboratory versus field conditions, or vice versa.

Development of remotely sensing devices (Cooke et al., 2004), as well as validation of point-of-care devices in fishes (Stoot et al., 2014), allows for field measurement of indicators once restrained to the laboratory. Specifically, with the advancement of biologging and biotelemetry technology, changes in activity levels and energetics (Burnett et al., 2014), foraging (Brownscombe et al., 2014), and heart rate (Clark et al., 2008) of free-swimming fish can be monitored before, during, and after a stressor (Donaldson et al., 2010; Raby et al., 2015). Limitations of this field-oriented mechanistic approach to measuring stress include cost, surgical requirements for tag implantation, detection efficiency, and tag retrieval, which can compromise study sample size. Measuring indicators in wild fishes naturally exposed to unpredictable, labile stressors (eg, cyclones, floods) can be incredibly revealing but is also highly opportunistic (Wingfield, 2013). A holistic, collaborative approach of field-based research coupled with complementary laboratory research focusing on mechanism(s) can provide the most complete assessment of the stress response.

4.6. Temporal Aspects

There are multiple levels of timing that can be considered when investigating animal stress. First, what life stage will the indicator of stress be measured? The hyporesponsive period early in development is well established for several species (eg, rainbow trout, Barry et al., 1995; Chinook salmon, *Oncorhynchus tshawytscha*, Feist and Schreck, 2001; yellow perch, *Perca flavescens*, Jentoft et al., 2002; lake sturgeon, *Acipenser fulvescens*, Zubair et al., 2012); endogenous stressor-induced cortisol production is not detected prior to hatch. Stressor-induced and resting plasma cortisol levels can shift (1) as sexual maturation progresses in salmonids (Pottinger and Carrick, 2000; Cook et al., 2011) and catfish (Barcellos et al., 2014b) and (2) during parental care in largemouth bass (Jeffrey et al., 2014b). Hyperresponsive periods are also present in smolting salmon (vs parr, Carey and McCormick, 1998). A longitudinal study by Koakoski et al. (2012) found that concentration and timing of peak stressor-induced plasma cortisol varied among fingerling, juvenile, and adult jundiá (*Rhamdia quelen*). To date, age effects largely focus on cortisol as the indicator of stress (Schreck and Tort, 2016; Chapter 1 in this volume, Figure 1.4). Life stage shifts in baseline levels of stress indicators may also confound quantification of stress. This caveat is relevant for other

physiological (eg, ontogeny of antioxidant defenses, Otto and Moon, 1996), performance (eg, rapid growth of larval coral reef species), and behavioral (eg, ontogeny of predator avoidance, Brown, 1984) stress indicators that can vary across life stage.

Second, what time of the day will the indicator of stress be measured? Resting heart rates (Aissaoui et al., 2000), plasma cortisol (Cousineau et al., 2014), and various behaviors (eg, activity, Bayarri et al., 2004) fluctuate on a diel cycle. Inconsistent timing of collection can skew data and comparison between studies with different sampling times can compromise validity of conclusions. It is noted, however, that some species may exhibit plasticity in traits typically associated with circadian rhythms (Reebs, 2002).

Third, what time poststressor will the indicator of stress be measured? As mentioned previously, time-course sampling is the most comprehensive approach to ensure capture of rise, peak, and recovery of the indicator. Induction and recovery times will vary, however, for different indicators (Gesto et al., 2015). Catecholamines (and other sympathetic nervous system processes such as heart rate and ventilation) are elevated instantaneously (seconds), whereas cortisol takes longer to elevate (minutes to hours) above prestressor levels. A lag between stressor exposure and changes in mRNA abundance (ie, transcription) is expected. Induction and recovery times may also vary depending on stressor type and severity (see Section 4.4). Identifying and describing temporal influences on the stress response is itself a topic of interest in stress biology; still, when treatment-level impacts (eg, stressor type or severity) are of interest, temporal influences should also be accounted for.

5. FROM INDIVIDUAL INDICATORS TO ECOSYSTEM HEALTH

Thus far this chapter has reviewed indicators of stress at the individual level, but an interesting extension to this work is to ask how the stress of an individual scales to population- and ecosystem-level processes. It is important to note that the molecular, physiological, and whole-animal indicators of stress described in this chapter are responsive over shorter timescales (minutes to days) relative to the response of populations and whole ecosystems to environmental stressors (months to years, see Figure 1 of Adams and Greeley, 2000). Carryover effects and intergenerational components of stress are especially integral when linking individual stress indicators to downstream population effects. Harsh overwintering conditions, episodes of low resource availability, and other environmental stressors can have carryover effects on a fish's phenotype even if stress

indicators suggest recovery (O'Connor et al., 2014; O'Connor and Cooke, 2015). Latent effects of stress on populations may be shaped by maternal match/mismatch (see Sheriff and Love, 2013; Love et al., 2013), and may not manifest for several generations. For example, using a 30-year dataset, Venturelli et al. (2010) found that maternally mediated effects on egg size in walleye (*Sander vitreus*) have the capacity to modulate population dynamics. Older, larger females produce larger eggs, which are apt to produce offspring with higher survival. Indeed, the authors detected higher population reproductive rates during years when older females were more abundant (Venturelli et al., 2010). If a stressor associated with a fisheries targeting older females compromises reproduction within this cohort, population stability could fluctuate via maternally mediated mechanisms (eg, changes in egg size, number, or energy content). Connecting individual stress indicators to larger scale processes can be achieved through modeling (Calow and Forbes, 1998; Fefferman and Romero, 2013) or correlation with population- and ecosystem-level metrics.

There are a number of commonly reported population- and ecosystem-level stress indicators including changes in population abundance, habitat use, age and size structure, sex ratios, and age at maturity (Shuter, 1990; Adams and Greeley, 2000; Bartell, 2006). At the ecosystem level, changes in indices of biotic integrity or species richness, food web structure, and productivity can all indicate environmental stress (Karr, 1981; Odum, 1985). Just as using multiple indicators is a robust way to define stress within an individual, an approach that incorporates many levels of biological organization to generate an ecosystem health assessment can be most informative (Attrill and Depledge, 1997; Adams and Greeley, 2000; Bartell, 2006; Yeom and Adams, 2007). For example, measuring indicators of thermal stress in captive adult salmon (eg, Jeffries et al., 2012) can be linked with fitness metrics of fishes migrating in the wild that are naturally experiencing higher water temperatures (Martins et al., 2012). These laboratory and field findings can then be scaled up to population and species survival trends using stock assessment data collected by government agencies. Changes in salmon population abundance can then be linked to health of other taxa (Bryan et al., 2013) and ecosystem-level processes (Gende et al., 2002). Connecting individual traits to an ecosystem is possible following pairing of laboratory recorded stress indicators of an individual, field-derived stress indicators, and population and ecosystem attributes amalgamated from an array of sources.

Drawing associations from individual to population or ecosystem, however, does require longitudinal datasets encompassing both individual-level stress indicators and field-based monitoring. Upon establishment that individual-level stress indicators correlate with population-level change, the more rapidly responding stress indicators can be utilized as early warning

signals of forthcoming population and ecosystem effects (Adams and Greeley, 2000). Focusing on sentinel or ecologically important organisms as bioindicators significantly aids in implementing individual stress profiles with assessments of ecosystem stability (Adams and Greeley, 2000). Such endeavors highlight the value and necessity of collaboration among academics, government, and user groups in order to develop stress indicators into useful tools for conservation and management.

6. STRESS INDICATORS OF THE FUTURE

Work outlined in this chapter, as well as in this volume, has provided a comprehensive understanding of how fish detect and respond to stressors. Despite the breadth in tools available to measure responses of fish to stressors (Fig. 11.2), and the wealth of questions that can be answered, there are a number of new directions that research in this area can take moving forward. With human populations projected to grow, and impacts to the planet anticipated to continue or intensify, improving our understanding of the response of fish to stressors, particularly in response to multiple stressors, is critical. We feel that there are five main areas that researchers should consider as targets for future work in hopes of both developing novel stress indicators and refining existing stress indicators to maximize benefits and predictive values.

First, and most importantly, there is a need to better link indices of stress and disturbance with metrics of reproductive output and fitness. The primary stress response is in itself a characteristic that is under selection (Wingfield et al., 1998; Ricklefs and Wikelski, 2002). Studies have used magnitude of the acute stress response as suggestion that populations may be at risk of experiencing declines (Romero and Wikelski, 2001). Despite the certainty of the relationship between chronic stress and reduced reproduction, links between activation of the acute stress response and fitness outcomes currently exist, to some extent, as correlative relationships rather than causative mechanisms. Identifying activation of the stress response, either following acute or chronic stressor exposure, does not necessarily guarantee that an animal will experience reduced fitness relative to unexposed animals. The ability to confidently link acute or chronic stress responses to reductions in fitness, or negative changes to other population parameters, would represent a monumental leap forward in our understanding of the importance of the stress response. As well, our ability to predict the outcomes of exposure to stressful stimuli and use of the cortisol stress response as an early warning system for conservation would be greatly

Figure 11.2. There are a growing number of stress indicators for use in fish, including some that are reasonably novel such as: (A) use of high throughput omic techniques; (B) use of point-of-care handheld meters for measuring blood chemistry in the field; (C) whole-body extraction of cortisol from small fish; (D) evaluation of reflex status; (E) assessment of swimming performance and metabolic status; and (F) evaluation of the locomotory activity and energetics of free-swimming fish using accelerometer biologgers. Photo credits: (A) Katrina Cook, (B) Steven Cooke, (C) Julia Redfern, (D) Vivian Nguyen, (E) Zach Zucherman, (F) Jacob Brownscombe.

enhanced. A metric relating stress to reproduction could also be coupled with the concept of carryover effects allowing the impacts of a current stressor across multiple reproductive bouts, or across generations. Indices linked to reproduction could then be associated with landscape-level stressors such as habitat modifications or climate change to discern the impact of these broad challenges on populations.

Second, similar to links between stress and fitness, we feel there is a need to strengthen links between stress and fish performance. The performance of a fish is a broad concept that includes metrics such as swimming ability, aerobic scope, and scope for a stress response (reactive scope). Links between these different metrics and outcomes such as survival and food acquisition are well established in the literature (Plaut, 2001; Farrell et al., 2008). However, stress and fitness/reproduction may also be linked indirectly through declines in organism performance (ie, decreased swimming performance can lead to reduced feeding and/or an inability to escape predation). There are a number of performance metrics or reflex impairments (eg, body flex, gag response) that correlate positively with stress and can be used to predict individual mortality (Davis, 2010). Because these reflex impairments can easily and reliably be collected in the field, it would be advantageous to link these performance metrics to outcomes beyond survival, including concepts such as reduced investment in reproduction or lowered fitness. Therefore, understanding how stress impacts organismal performance, over both short and long terms, can aid in our ability to predict impacts of stress on individuals, and, ultimately, on populations.

Third, to facilitate connection between individual stress indicators and population-level processes, there is a need to further our understanding of the indicators mediating intergenerational effects. Egg size, fertilization success, and embryonic survival are established indicators of parental stress. Accompaniment of these metrics with the evaluation of physiological and behavioral traits of progeny is becoming more prevalent, and can reveal latent indicators of intergenerational stress. Still presently lacking is knowledge of gametic stress indicators driving changes to offspring phenotype. Elevated levels of egg cortisol are thought to be a reliable stress indicator that also serves as a mechanism of offspring change (Gingerich and Suski, 2011). However, whether maternal stressor exposure alters cortisol levels in eggs remains equivocal (eg, Stratholt et al., 1997 and Sopinka et al., 2014). Further experimentation is required to confirm if concentration of egg cortisol (or other hormones such as thyroid and sex steroids) is a reliable indicator of maternal stress. Also, with the advancement of molecular technologies (see later), quantification of epigenetic changes in the transcriptome of eggs, embryos, and sperm (Cabrita et al., 2014; Mommer and Bell, 2014) has the potential to serve as a

valuable stress indicator. Expanding the repertoire of reproductive-based stress indicators will aid in predicting the cascading effects of stress from one generation to the next.

Fourth, research has recently demonstrated that stress hormones can be deposited and archived in structures such as fur or feathers (Bortolotti et al., 2009; Sheriff et al., 2011). The ability to extract stress hormones from structures such as fur or feathers provides a unique, long-term, integrated history of the activity of the stress axis, and can serve as a catalog of past events in the life of an animal. In addition, these structures can be collected nonlethally, and are often freely shed by animals. At present, we are aware of a single study that has measured stress hormones (cortisol) from scales (Aerts et al., 2015), demonstrating the potential to use elasmoid scales as a stress biomarker for fishes. We would encourage exploration into the area of cortisol deposition in scales as this could provide a valuable tool for both nonlethally defining the stress history of a free-swimming animal, and potentially relating stressful events in the past to reproductive output or fitness.

Finally, the last decade has seen the emergence of a number of new technologies for quantifying the molecular responses of animals to various disturbances, including tools such as transcriptomics, gene expression, and protein generation, which has provided a powerful new way to quantify how organisms interact with their environment (Evans and Hofmann, 2012). These techniques can provide reliable indices of stress, assay a number of different physiological systems simultaneously, and importantly, link changes in gene expression to ecologically relevant outcomes such as survival and fitness (Abzhanov et al., 2006). Fish are ideally suited for studies using these molecular tools because they possess a number of different nucleated tissues that can be collected nonlethally (eg, gills, Jeffries et al., 2014a; red blood cells, Dennis et al., 2015). These tools become particularly valuable when they are linked to whole-organism metrics of performance, intergenerational effects (ie, epigenetics), landscape-level challenges, or demographic patterns to define population-level trends, rather than simply cataloging the stress response of an animal. We therefore encourage the continued development and proliferation of these novel tools.

7. CONCLUSION

Exposure of fish to a stressor evokes activation of the HPI axis (primary response) and subsequent secondary and tertiary responses. Both HPI axis activity and whole-animal responses are quantified to indicate stress (see Section 3 and Tables 11.1–11.3). Quantification of stress under laboratory

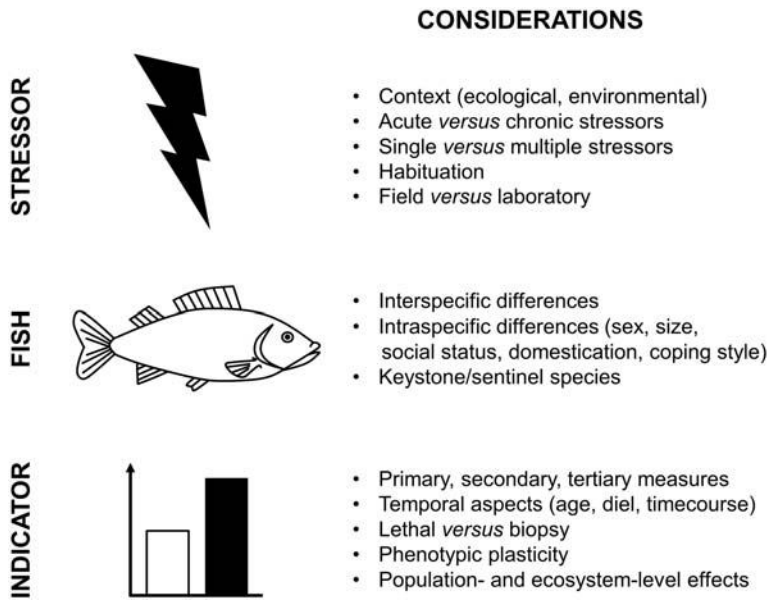

Figure 11.3. Considerations when quantifying and interpreting indicators of stress in fishes.

and field conditions is fundamental to understanding how a fish responds to changes in its internal and external environment. This knowledge is continuing to be implemented into the management and conservation of wild populations, as well as maintaining welfare of captive fishes. The number of different stress indicators reported in the literature is matched by the number of different factors that must be considered when quantifying and interpreting the indicators themselves (Fig. 11.3). Be it the severity or contemporaneous nature of the stressors, the variation in response within populations and among species, or the time of day the indicator is measured, our understanding of stress is challenged by many subtle and significant variables. The future of stress indicators will entail a combination of (1) optimizing experimental approaches to ensure indicators are reliable and ecologically-relevant; (2) examining stress across levels of organization within individuals (ie, molecular to whole-animal responses), as well as integrating individual responses with population- and ecosystem-level stress indices; (3) accounting for and determining indicators relevant for carryover and intergenerational effects; (4) enhancing new technologies such as biologging, telemetry, and noninvasive assessments of genomic and endocrine stress profiles; and (5) continued efforts to collaborate among disciplines.

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