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NOTE

Are 3 minutes good enough for obtaining baseline physiological samples from teleost fish?

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Abstract: A prerequisite to studying the physiological status of wild animals is the ability to obtain blood samples that reflect the condition prior to capture or handling. Based on research in avian taxa, it is recommended that such samples be obtained within 3 min of capture; however, this guideline has not been validated in wild teleosts. The present study addresses the time course of physiological changes in a number of blood metrics across six species of freshwater fish. Fishes were caught using a standardized angling protocol and held in a water-filled trough prior to the collection of a blood sample, via caudal phlebotomy, between 0.5 and 11 min after capture. Changes in whole-blood glucose and lactate concentrations, hematocrit, and plasma cortisol concentrations were assessed. Change-point analyses indicated that blood lactate concentrations and hematocrit did not deviate from baseline values until ~2–5 min of handling for all species, whereas blood glucose concentrations generally did not deviate significantly from baseline over the 11 min test period. In all species, plasma cortisol concentrations began to increase above baseline between ~4 and 8 min after capture. Thus, to ensure that blood samples are representative of baseline conditions across multiple metrics, we recommend that sampling be limited to less than 2 min in teleost fishes.

Key words: stress, cortisol, field endocrinology, glucose, lactate, blood sampling, hematocrit, 3 min rule, wild animals, teleost, largemouth bass, *Micropterus salmoides*, smallmouth bass, *Micropterus dolomieu*, rock bass, *Ambloplites rupestris*, pumpkinseed, *Lepomis gibbosus*, bluegill, *Lepomis macrochirus*, northern pike, *Esox lucius*.

Résumé : Une condition préalable à l'étude de l'état physiologique d'animaux sauvages est la capacité d'obtenir des échantillons de sang qui reflètent l'état avant la capture ou la manipulation. À la lumière de travaux de recherche sur des taxons d'oiseaux, il est recommandé que de tels échantillons soient obtenus dans les 3 min suivant la capture. Cette directive n'a toutefois pas été validée pour les téléostéens vivant à l'état sauvage. L'étude s'est penchée sur la chronologie de changements physiologiques pour différents paramètres sanguins pour six espèces de poissons d'eau douce. Les poissons ont été capturés en suivant un protocole normalisé de pêche à la ligne et maintenus dans une cuve remplie d'eau avant le prélèvement d'un échantillon de sang par ponction caudale, de 0,5 à 11 min après la capture. Les variations des concentrations de glucose et de lactate du sang entier, de l'hématocrite et des concentrations de cortisol plasmatique ont été évaluées. Les analyses des points de changement indiquent que les concentrations de lactate du sang et l'hématocrite ne divergent pas des valeurs de référence avant ~2–5 min de manipulation pour toutes les espèces, alors que les concentrations de glucose du sang ne divergent pas significativement, en général, des valeurs de référence pendant toute la période de manipulation de 11 min. Chez toutes les espèces, la concentration de cortisol plasmatique commence à augmenter au-delà de la valeur de référence entre ~4 et 8 min après la capture. Ainsi, pour s'assurer que les échantillons de sang soient représentatifs des conditions de référence pour différents paramètres, nous recommandons que leur prélèvement soit fait dans les 2 min suivant la capture chez les poissons téléostéens. [Traduit par la Rédaction]

Mots-clés : stress, cortisol, endocrinologie de terrain, glucose, lactate, prélèvement sanguin, hématocrite, règle de 3 minutes, animaux sauvages, téléostéen, achigan à grande bouche, *Micropterus salmoides*, achigan à petite bouche, *Micropterus dolomieu*, crapet de roche, *Ambloplites rupestris*, crapet-soleil, *Lepomis gibbosus*, crapet arlequin, *Lepomis macrochirus*, grand brochet, *Esox lucius*.

Introduction

In many vertebrate species, the physiological dynamics of blood have served as an important tool in assessing whole-organism responses to environmental change and stressors. For example, acute increases in cortisol levels are often associated with stressor exposure as a direct product of stress axis activation (i.e., the hypothalamic–pituitary–interrenal (HPI) axis; Barton 2000; Jentoft et al. 2005; Lawrence et al. 2015; reviewed in Wendelaar Bonga (1997) and Gorissen and Flik (2016)), with cortisol being the primary glucocorticoid stress hormone in teleost fish. As a result, blood analyses have been and continue to be a central component of a large proportion of the physiological literature pertaining to teleost fish (Stoot et al. 2014).

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A central aim of contemporary fish physiology is to characterize how fish respond to environmental and anthropogenic stressors (e.g., Conte 2004; Baker et al. 2013; Schulte 2014). As with other vertebrates, teleost fishes employ both the HPI axis and the sympathetic axis to initiate physiological and behavioural responses to environmental challenges. The actions of these axes assist in the maintenance and (or) re-establishment of internal homeostasis and are generally focused on re-prioritizing energy distribution, engaging de novo synthesis of high energy substrates, and maintaining ionic and osmotic balance (Wendelaar Bonga 1997; Mommsen et al. 1999; Gorissen and Flik 2016). The blood plays a central role in these processes by transporting hormones (e.g., cortisol, catecholamines). As well, the shuttling of energetic substrates and stress-induced hydromineral imbalances are reflected in the milieu of the blood's physical chemistry. Commonly used biomarkers of stress include circulating concentrations of glucose, lactate, cortisol, and inorganic ions (e.g., Na^+, K^+, Cl^-, Ca^{2+}), as well as hematocrit (Suski et al. 2007; Arlinghaus et al. 2009; Jeffries et al. 2012; reviewed in Sopinka et al. (2016)).

Baseline metrics are an important consideration in assessing the impacts of a stressor (Sopinka et al. 2016). Establishing baseline blood parameters requires that blood be collected from the animal quickly and with minimal disturbance. In most instances, caudal venipuncture is employed as the primary collection method (e.g., Suski et al. 2007; Jeffries et al. 2012; Lawrence et al. 2015), but this technique requires that the animal be handled. Handling stress effects have been well characterized across a number of teleost species and alter the blood physiology of the animal with respect to both energetic and hydromineral balances (Cleary et al. 2000; Shrimpton et al. 2001; Morales et al. 2005; reviewed in Pankhurst (2011)). Thus, haste in blood sampling is of critical importance in establishing representative and accurate baseline values, which begs the question: how long is too long?

It is important to establish sampling times from which the baseline physiology of the fish can be reliably assessed. In the avian literature, Romero and Reed (2005) tested the widely held view that a maximum handling time of 3 min was sufficient to capture baseline titres of corticosterone. These authors concluded that sampling should occur within 2 min of capture, with 3 min being the uppermost limit (Romero and Reed 2005). Small et al. (2017) came to similar conclusions (~2 min). Within the teleost literature, most blood samples appear to be collected within 3 min of the animal's capture (e.g., Knapp and Neff 2007; Cook et al. 2012). However, the assumption that this "3 min rule" delivers a blood sample that is representative of baseline conditions does not appear to have been tested for teleost fish. Furthermore, information on the acute time course (i.e., minutes post stressor) of the effects of handling stress on teleost blood physiology appears to be lacking in the current literature. Thus, the purposes of the present study were to (*i*) establish a time course, over an acute time frame, of the physiological changes associated with handling typical of a blood sampling event for wild teleost fish, (*ii*) identify the time at which blood physiological status deviates from baseline levels, and (*iii*) establish guidelines for the timing of baseline blood samples in teleost fishes.

Materials and methods

Study site and species

All fish used in this study were collected from Lake Opinicon (44.5590°N, 76.3280°W; Chaffey's Lock, Ontario, Canada), a shallow and eutrophic freshwater lake in eastern Ontario, Canada. Study species were selected based on their widespread use in fisheries science and their importance to recreational sport fisheries (reviewed in Cooke and Philipp (2009)). The selected species were largemouth bass (*Micropterus salmoides* (Lacepède, 1802)), smallmouth bass (*Micropterus dolomieu* Lacepède, 1802), rock bass (*Ambloplites rupestris* (Rafinesque, 1817)), bluegill (*Lepomis macrochirus* Rafinesque, 1819), pumpkinseed (*Lepomis gibbosus* (Linnaeus, 1758)), and northern pike (*Esox lucius* Linnaeus, 1758). Capture occurred between the months of May and July of 2015 via rod and reel angling (see below). Centrarchid fishes except smallmouth bass were generally captured from shallow, weedy bays (<2.5 m depth), whereas northern pike and smallmouth bass were captured from the deeper, cooler reaches of the lake (~3–8 m depth). Mean surface water temperature was 21.5 ± 0.8 °C during the course of this experiment.

Experimental series

Fishes were captured using a variety of artificial lures and recreational fishing rod and reel setups. To control for physiological disturbances associated with angling itself, the timing of angling events was standardized so that each animal was played on the line for 20 s following a successful hookset. Control fish were an exception to this protocol; here, the animal was immediately brought on board with hookset-to-landing times of less than 10 s. Upon landing on the boat, the animal was promptly placed on its dorsal side in a water-filled trough and immobilized by a deck hand. During this time, the hook was removed from the animal. The handling procedures described above attempted to replicate the typical treatment of a fish during blood sampling events in a standard field assessment (e.g., where fish are hot-picked from a gill net, dip netted, or caught by hook and line).

Individual fish were bled once, at a time ranging between 30 s and 11 min of total handling time; these times were chosen to encompass a range realistic for field settings. Handling time was calculated as the total time elapsed from hookset to blood withdrawal. Blood was collected by caudal phlebotomy using a heparinized (10 000 USP units·mL^{-1} Na^+ heparin; Sandoz, Boucherville, Quebec, Canada) 1 mL syringe with a 23-gauge needle. Blood was immediately assessed for whole blood glucose and lactate concentrations ([glucose] and [lactate], respectively), as well as hematocrit. Thereafter, blood was held (1–2 h maximum) in a water–ice slurry until centrifugation (5 min at 2000*g*; Mandel Scientific, Guelph, Ontario, Canada). The plasma was decanted, flash frozen, and stored at −80 °C for subsequent determination of plasma cortisol concentrations ([cortisol]). Following blood sampling, each fish was measured for total length (to the nearest millimetre) and promptly released back into the lake.

Blood and plasma analyses

Plasma [cortisol] was assessed using a commercial radioimmunoassay kit (RIA; ImmuChem Cortisol Coated Tube RIA Kit, MP Biomedicals, Solon, Ohio, USA). This assay has been validated for use in teleost fishes (Gamperl et al. 1994) and has been used previously in a number of the species studied here (Cook et al. 2012; McConnachie et al. 2012; Zolderdo et al. 2016). Intra- and inter-assay coefficients of variation for the cortisol RIA were 6.9% and 12.0%, respectively. Blood [glucose] and [lactate] were determined using portable, medical-grade glucose (Accu-Chek Compact Plus, Hoffman-La Roche Limited, Mississauga, Ontario, Canada) and lactate (Lactate Plus, Nova Biomedical Corporation Canada Ltd., Mississauga, Ontario, Canada) meters, respectively. These units have been validated for use in teleost fishes (reviewed in Stoot et al. (2014)). Hematocrit was measured by collecting blood into heparinized, microcapillary tubes (ammonium heparin; Drumond Scientific Co., Broomall, Pennsylvania, USA) that were centrifuged (13 700*g* for 5 min; StatSpin CritSpin, Beckman Coulter, Brea, California, USA).

Statistical analyses

SigmaPlot (version 11.0; Systat Software Inc., San Jose, California, USA) was used to conduct all regression analyses and to calculate the descriptive statistics for the baseline blood parameters reported in Table 1. "Baseline" parameters represent a simple mean of the values for the control animals subjected to minimal

Table 1. Average total length and the size ranges for six species of wild teleost fish.

Species	Total length (mm)	Minimum length (mm)	Maximum length (mm)
Largemouth bass (*Micropterus salmoides*)	309±5 (*N* = 66)	235	415
Smallmouth bass (*Micropterus dolomieu*)	339±11 (*N* = 26)	240	430
Rock bass (*Ambloplites rupestris*)	201±4 (*N* = 48)	160	250
Bluegill (*Lepomis macrochirus*)	161±3 (*N* = 66)	121	203
Pumpkinseed (*Lepomis gibbosus*)	187±2 (*N* = 68)	139	220
Northern pike (*Esox lucius*)	547±15 (*N* = 39)	325	735

Note: Values are presented as means ±1 SEM; *N*, the number of samples.

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angling, as described above. Multiple linear regressions were employed to test for the relationships among the physiological parameter of interest, sampling time, and total length. In cases where a change-point value was determined (see below), individual regressions were plotted for the phases before and after the change point. If no change point was evident, regression lines were fitted to the entire dataset. Simple linear regressions were used to test for relationships between physiological parameters. Logarithmic transformations were used to transform non-normally distributed data used in regression analyses. For visual simplicity, regression data are plotted as the relationship between the blood metric of interest and time (i.e., ignoring animal length). To identify the change point for each parameter, the package "changepoint" in R (version 2.2.2; Killick et al. 2016) was used. The change-point value represents the time at which the dataset undergoes a statistically significant deviation from the previous state (Siegel and Castellan 1988; Romero and Reed 2005). All statistical evaluation was conducted at $\alpha = 0.05$, with data being presented, as appropriate, as mean ±1 standard error of the mean (SEM) and sample size (*N*).

Animal ethics

All procedures were conducted in accordance with the guidelines for the use of animals in research and teaching of the Canadian Council on Animal Care, under the administration of the Carleton University Animal Care Committee (AUP #104288).

Results

Fish body size data are reported in Table 1.

Cortisol

Baseline plasma [cortisol] values ranged from 1.68 to 2.85 ng·mL^{-1} (Table 2), and change-point analysis indicated that for most species, plasma [cortisol] did not deviate from this baseline state until approximately 6.5 min post capture (Table 3; Fig. 1). The exception was northern pike, which exhibited a change point at 4.3 min post capture (Fig. 1). Owing to an unfortunate issue associated with sample storage, all but a handful of plasma samples for smallmouth bass were lost and therefore no data are reported for plasma [cortisol] for this species.

Prior to the change point, plasma [cortisol] increased with handling time in largemouth bass ($p < 0.001$), rock bass ($p = 0.021$), bluegill ($p < 0.001$), pumpkinseed ($p < 0.001$), and northern pike ($p = 0.005$); see Table 4 for r^2 data for all linear relationships. After the change point, plasma [cortisol] was not significantly affected by time ($p > 0.05$) in any species. Plasma [cortisol] levels after change point were quite variable among species, with bluegill and pumpkinseed appearing to mount the greatest cortisol responses (peak values ~300–400 ng·mL^{-1}). Fish length was not correlated with plasma [cortisol] in any species ($p > 0.05$).

Blood glucose

Baseline blood [glucose] never exceeded 3.0 mmol·L^{-1} (Table 2). For most species, significant deviations of blood [glucose] from the baseline state were not observed within the experimental time frame (Fig. 2). However, smallmouth bass and pumpkinseed exhibited significant change-point times at 4.8 and 8.7 min, respectively (Table 3; Fig. 2).

Increases in handling time led to significant increases in blood [glucose] in largemouth bass ($p < 0.001$), rock bass ($p = 0.015$), bluegill ($p < 0.001$), and northern pike ($p < 0.001$) (Fig. 2). However, handling time alone was not able to account for the majority of the variation (i.e., $r^2 < 0.417$) observed in blood [glucose] in these species. For both smallmouth bass and pumpkinseed, blood [glucose] was not related to handling time either before or after change point ($p > 0.05$; Fig. 2). Blood [glucose] was not related to body length for any species ($p > 0.05$) except northern pike, where larger fish had lower blood [glucose] relative to smaller individuals ($p < 0.001$).

Blood [glucose] was related to, in a significant, positive manner, plasma [cortisol] in largemouth bass ($p < 0.001$), northern pike ($p = 0.017$), pumpkinseed ($p < 0.001$), and bluegill ($p < 0.001$) (Fig. 3). However, plasma [cortisol] explained only a small (i.e., 14%–40%) proportion of the variation in blood [glucose] (Fig. 3). In rock bass, there was no apparent relationship between these two parameters ($p = 0.598$; Fig. 3).

Blood lactate

Baseline blood [lactate] were below 0.8 mmol·L^{-1} in all species (Table 2). Deviations from baseline blood [lactate] occurred over a span of 2.5 to 3.8 min for all species, making this blood metric the most sensitive (i.e., most time responsive) measured (Table 3; Fig. 4).

Prior to the change point, blood [lactate] was significantly related to time in all species (largemouth bass, $p = 0.031$; smallmouth bass, $p = 0.035$; bluegill, $p = 0.007$; pumpkinseed, $p = 0.032$; and northern pike, $p = 0.038$) except rock bass ($p = 0.135$; Fig. 4). Similarly, blood [lactate] was significantly related to time after change point for all species (largemouth bass, $p < 0.001$; rock bass, $p = 0.008$; bluegill, $p = 0.015$; and northern pike, $p < 0.001$) except smallmouth bass ($p = 0.533$) and pumpkinseed ($p = 0.833$; Fig. 4). In northern pike, blood [lactate] was significantly related to body length ($p = 0.009$) during the phase after change point only, where larger individuals had lower blood [lactate].

Hematocrit

Baseline haematocrits ranged from 16% to 26% (Table 2), and change-point times were quite variable among the species studied (Table 3; Fig. 5). Hematocrit was most sensitive to sampling time in bluegill and pumpkinseed, where haematocrit increased above baseline values at 2.9 and 2.0 min, respectively. Northern pike exhibited intermediate sensitivity, with a change point of 3.48 min. Hematocrit was slowest to respond to sampling time in largemouth, smallmouth, and rock bass, with change points of 6.8, 5.1, and 4.6 min, respectively.

Hematocrit was not significantly affected by time for any species or time period (Fig. 5). Overall, the relationship between handling time and hematocrit was weak, with <10% of the variation in hematocrit being explained by handling time. Hematocrit levels were unaffected by body length for all species.

Table 2. Baseline blood parameters for six species of wild teleost fish.

Species	Plasma [cortisol] (ng·mL^{-1})	Blood [glucose] (mmol·L^{-1})	Blood [lactate] (mmol·L^{-1})	Hematocrit (%)
Largemouth bass (*Micropterus salmoides*)	2.85±0.22 (*N* = 11)	2.2±0.1 (*N* = 12)	0.71±0.3 (*N* = 12)	22.6±1.0 (*N* = 12)
Smallmouth bass (*Micropterus dolomieu*)	—	2.7±0.1 (*N* = 6)	0.23±0.2 (*N* = 6)	26.3±2.9 (*N* = 5)
Rock bass (*Ambloplites rupestris*)	2.16±0.30 (*N* = 5)	2.7±0.1 (*N* = 6)	0.35±0.2 (*N* = 6)	22.4±1.3 (*N* = 4)
Bluegill (*Lepomis macrochirus*)	2.07±0.30 (*N* = 5)	2.5±0.1 (*N* = 6)	0.20±0.1 (*N* = 6)	20.3±1.4 (*N* = 6)
Pumpkinseed (*Lepomis gibbosus*)	1.68±0.20 (*N* = 3)	2.9±0.2 (*N* = 5)	0.68±0.2 (*N* = 5)	21.9±2.5 (*N* = 4)
Northern pike (*Esox lucius*)	2.81±0.77 (*N* = 4)	2.7±0.3 (*N* = 7)	0.79±0.1 (*N* = 7)	16.6±1.7 (*N* = 7)

Note: Values are presented as means ±1 SEM; *N*, the number of samples.

Table 3. Change-point times (in minutes) for blood parameters measured in six species of teleost fish subjected to a handling stress of 0 to 11 min duration.

	Change-point times (min)			
Species	Plasma [cortisol]	Blood [glucose]	Blood [lactate]	Hematocrit
Largemouth bass (*Micropterus salmoides*)	6.6	—	2.8	6.8
Smallmouth bass (*Micropterus dolomieu*)	ND	4.8	3.5	5.1
Rock bass (*Ambloplites rupestris*)	7.8	—	3.8	4.6
Bluegill (*Lepomis macrochirus*)	7.5	—	2.8	2.9
Pumpkinseed (*Lepomis gibbosus*)	7.9	8.7	2.5	2.0
Northern pike (*Esox lucius*)	4.3	—	3.5	3.5

Note: Values represent the time at which a change in the parameter from its baseline state is evident. Parameters where a change point was not detected are denoted by "—". No data were available for plasma cortisol concentrations in smallmouth bass (ND).

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Discussion

Corticosteroid responses

Cortisol biosynthesis is regulated by the higher centres of the HPI axis in teleost fish, and the baseline level of cortisol production is important in fine-scale regulation of metabolism (Van Der Boon et al. 1991; Romero et al. 2009; Gorissen and Flik 2016). In wild and captive or domesticated fish, baseline [cortisol] typically ranges from 2 to 30 ng·mL^{-1} (reviewed in Barton (2002) and Pankhurst (2011)). The baseline [cortisol] are within the "unstressed" range for the species measured here (Suski et al. 2003; McConnachie et al. 2012; Pullen et al. 2017), demonstrating that appropriate sampling procedures can yield baseline values even in teleost fish captured from the wild.

In response to a stressor, the HPI axis is activated, eliciting an increased rate of cortisol biosynthesis and culminating in elevated titres in circulation (~40–600 ng·mL^{-1}; Suski et al. 2003; Cook et al. 2012; Pullen et al. 2017), as observed in all species examined in the present study. Under these conditions, cortisol acts to increase energetic substrate availability and restore hydromineral balance (reviewed in Mommsen et al. (1999) and Schreck and Tort (2016)). Changes in circulating cortisol titres require time, with cortisol titres in the species studied here not deviating from baseline levels until at least 4 min into the handling exposure, and peak values typically occurring ~30–40 min after stressor (Pickering et al. 1982; Cook et al. 2012). Because cortisol titres do not rise immediately upon exposure to a stressor, the 3 min guideline has long been considered adequate to capture representative baseline cortisol titres in a number of vertebrate taxa (reviewed in Romero and Reed (2005)). Romero and Reed (2005) demonstrated the validity of this "rule" experimentally using a number of bird species; cortisol titres were preserved at baseline within 3 min of sampling time (see also Wingfield et al. 1982; Sockman and Schwabl 2001). In studies of teleost fish, the current body of literature suggests that the timing of cortisol responses is variable across species, with baseline cortisol titres being observed up to 15 min after acute stressor exposure in some fish (Kubokawa et al. 2001), although 5 to 10 min post stressor is more common (Barton et al. 1980; Sumpter et al. 1986; Pankhurst et al. 1992; Kubokawa et al. 2001). In agreement with previous findings, cortisol titres rose above baseline ~4–8 min after stressor in all species presented here. Thus, for a temperate teleost fish, the 3 min rule is valid and allows for response variation reflecting the impact of factors, including stress history (reviewed in Barton (2002)), age (Koakoski et al. 2012), and body condition and size class (Cook et al. 2012), on cortisol dynamics.

Glucose responses

In contrast to mammals, which tend to regulate blood [glucose] within a narrow range, baseline blood [glucose] varies considerably with taxonomic group, feeding guild, and feeding status in teleosts (Moon 2001; Polakof et al. 2012). Baseline [glucose] in the present study were comparable to values reported previously for wild centrarchid fishes (Thompson et al. 2008; McConnachie et al. 2012; Zolderdo et al. 2016; reviewed in Kieffer and Cooke (2009)), although values for northern pike were somewhat lower than previously reported (~3–6 mmol·L^{-1}; Ince and Thorpe 1975; Schwalme and Mackay 1985; Arlinghaus et al. 2009; Pullen et al. 2017).

Alterations in blood [glucose] are widely used as a metric of a vertebrate's stress status (Boonstra 2004; Romero and Butler 2007; Sopinka et al. 2016). For example, in teleost fishes, a variety of acute stressors elicits an increase in blood [glucose] (Jentoft et al. 2005; Suski et al. 2007; Lawrence et al. 2017), as was apparent in most of the species presented here, where blood [glucose] increased through handling time. Elevations in [glucose] serve to meet the heightened energetic demands during a stress response (Mommsen et al. 1999; Barton and Iwama 1991) and are regulated under the HPI axis via cortisol and the sympathetic axis via catecholamines. Catecholamines elevate blood [glucose] by promoting glycogen breakdown and, to a lesser extent, stimulating gluconeogenic capacity (i.e., de novo glucose synthesis; Perry and Capaldo 2011). Cortisol acts mostly to upregulate gluconeogenic pathways (Mommsen et al. 1999). Here, the observed rise in blood [glucose] across all species likely represents a contribution of both stress axes, an observation supported by the fact that the relationship between plasma [cortisol] and blood [glucose] explained ≤40% of the variation in blood [glucose]. Catecholamine-mediated responses in blood [glucose] have been seen in other sportfish species (Ling and Wells 1985; Lowe and Wells 1996; reviewed in Cooke et al. (2013*a*)).

Fig. 1. Relationship between plasma cortisol concentration (ng·mL^{-1}) and handling time (min) for six freshwater teleost species. The time at which plasma [cortisol] diverged from baseline status (i.e., the change point) is indicated by the vertical dotted line. Regression lines were fitted before (solid circles; N = 16–36) the change-point value; no significant relationships were detected after (open circles; N = 11–21) the change-point value. Significant relationships between plasma [cortisol] and handling time in the phase before the change point were found in (A) largemouth bass (*Micropterus salmoides*; [cortisol] = 4.52 + (2.34 × time) – (0.02 × length); $p < 0.001$; $r^2 = 0.281$), (C) rock bass (*Ambloplites rupestris*; [cortisol] = 8.47 + (1.25 × time) – (0.04 × length); $p = 0.021$; $r^2 = 0.132$), (D) bluegill (*Lepomis macrochirus*; [cortisol] = 54.90 + (14.91 × time) – (0.48 × length); $p < 0.001$; $r^2 = 0.314$), (E) pumpkinseed (*Lepomis gibbosus*; [cortisol] = –13.59 + (14.34 × time) – (0.05 × length); $p < 0.001$; $r^2 = 0.368$), and (F) northern pike (*Esox lucius*; [cortisol] = 0.72 + (2.19 × time) – (3.98 × 10^{-3} × length); $p = 0.005$; $r^2 = 0.400$). Only six cortisol samples for (B) smallmouth bass (*Micropterus dolomieu*) were retrieved and they are presented for illustration only. Scaling of the y axes has been adjusted for each species to reflect the maximum [cortisol].

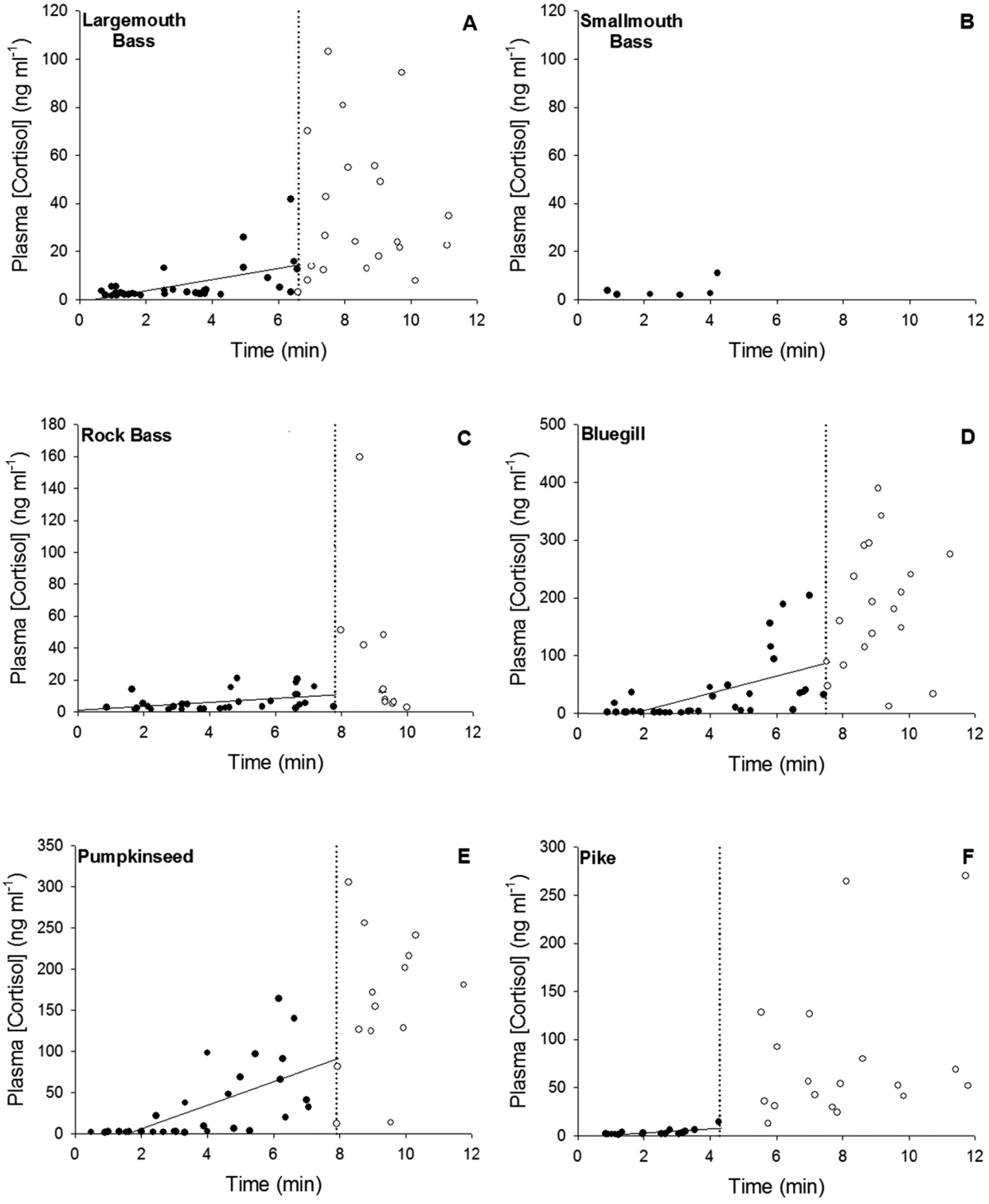

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Table 4. Correlation coefficient (r^2) data for the linear regression plots of all parameters measured for all species.

	Plasma [cortisol]		Blood [glucose]		Blood [lactate]		Hematocrit	
Species	Pre r^2	Post r^2	Pre r^2	Post r^2	Pre r^2	Post r^2	Pre r^2	Post r^2
Largemouth bass (*Micropterus salmoides*)	0.281	0.000	0.372	—	0.023	0.367	0.051	0.015
Smallmouth bass (*Micropterus dolomieu*)	ND	ND	0.000	0.0147	0.474	0.118	0.000	0.187
Rock bass (*Ambloplites rupestris*)	0.132	0.289	0.134	—	0.030	0.178	0.000	0.090
Bluegill (*Lepomis macrochirus*)	0.314	0.000	0.411	—	0.298	0.111	0.000	0.025
Pumpkinseed (*Lepomis gibbosus*)	0.368	0.000	0.062	0.053	0.246	0.000	0.099	0.000
Northern pike (*Esox lucius*)	0.400	0.000	0.417	—	0.168	0.479	0.000	0.000

Note: Pre, before change point; Post, after change point. In the instance where there was a singular regression line (i.e., no time frame for before or after the change point), the value for the relationship can be found in the "Pre r^2" column with a "—" in the "Post r^2" column.

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In most of the species investigated here, there appeared to be no change-point time for blood [glucose], with blood [glucose] gradually rising with handling time to reach values ~200% of baseline. While information is limited, comparable responses have also been observed in other teleost species (Perrier et al. 1978; Schwalme and Mackay 1985; Thomas and Robertson 1991; Waring et al. 1996; O'Toole et al. 2010). Given the relatively slow pace of the rise in blood [glucose] through time, reliable baseline values could reasonably be captured within 3 min of a sampling event. Interestingly, pumpkinseed and smallmouth bass were the only two species to demonstrate a change point in blood glucose titres, occurring at 8.7 and 4.8 min, respectively. The 3 min time frame would still incorporate the observations for these two species, thereby providing a conservative time frame for assessing baseline blood glucose physiology.

Lactate responses

Because anaerobic metabolism results in the formation of lactic acid, lactate's presence in the blood is a valuable metric in determining an organism's aerobic status, serving as a proxy of tissue-level oxygen availability (Livingstone 1983), in addition to the sustained use of anaerobic muscle fibres (i.e., burst swimming; Wood 1991; Wells and Baldwin 2006). Under conditions of sufficient oxygen availability and limited fight times, baseline values of lactate in the blood should be low, as seen in most resting centrarchid fishes (<1 mmol·L^{-1}; Furimsky et al. 2003;Suski et al. 2003). Similarly, even with an acute angling event, blood lactate levels in the fish of the present study were below 1 mmol·L^{-1}, suggesting minimal accumulation of an oxygen debt at the time of sampling (Wood 1991). However, change-point analyses indicated that lactate levels rose above baseline within 2.5–4 min of handling, making blood lactate the most time-sensitive metric examined in the present study. Elevated lactate titres in the blood likely represented a combination of reduced oxygen uptake at the gills (i.e., air exposure) and exercise-induced oxygen debt (i.e., burst swimming) associated with angling (Perrier et al. 1978; Furimsky et al. 2003; Suski et al. 2007; Brownscombe et al. 2014). Indeed, the time course for blood lactate observed here parallels that in other teleost fishes (Perrier et al. 1978; Currey et al. 2013; O'Toole et al. 2010; reviewed in Wood (1991)). Smallmouth bass were unique in lacking a significant relationship between time and blood lactate levels, perhaps a reflection of the high physiological performance of this species relative to other centrarchids (Kieffer and Cooke 2009), as well as the minimal fight intensity exhibited by these animals (personal observations). Northern pike were also unusual in that lactate levels were inversely related to body length. A similar relationship was reported previously in largemouth bass (Kieffer et al. 1996) and may represent a size-dependent hydrological effect as proposed by Kieffer and Cooke (2009).

Given the high sensitivity of blood lactate levels to the combination of angling and handling, it is recommended that baseline sampling for this parameter be conducted within 2 min; longer durations run a high risk of obtaining a sample that is not clearly baseline. It should also be noted that blood lactate level is highly correlated with fight time and intensity during an angling event (O'Toole et al. 2010; Brownscombe et al. 2014; reviewed in Kieffer and Cooke (2009)) and, consequently, angling duration should be minimized or standardized to capture representative baseline values for blood lactate. Finally, it is worth reiterating that blood lactate is not a stress-related metric in teleost fish but rather reflects oxygen availability, which operates independently of the HPI axis (Livingstone 1983; Wood 1991).

Hematocrit responses

Baseline hematocrit was within the reported range for other centrarchid species (Furimsky et al. 2003; McConnachie et al. 2012; Ward et al. 2017) and esociformes (Colotelo et al. 2013; Pullen et al. 2017). Traditionally, hematocrit has been used as a metric of respiratory distress because it often increases under conditions of limited oxygen availability (Randall 1982; Fange 1992). In teleosts, increases in hematocrit can arise from splenic contractions, thus increasing the total number of red cells in circulation (Pearson and Stevens 1991; Lai et al. 2006; reviewed in Randall and Perry (1992)), and (or) through an osmotic effect resulting from lactate accumulation in the muscle (i.e., "hemoconcentration"; Pearson and Stevens 1991; Caldwell et al. 2006; reviewed in Wood and Perry (1985)). The significant relationship between blood [lactate] and hematocrit for all centrarchid species measured here provides support for this contention. Splenic release of red cells increases the oxygen carrying capacity of the blood to compensate for systemic oxygen shortfalls (Wells et al. 2003) and is regulated by catecholamines (Randall and Perry 1992). The rapid mobilization of catecholamines in response to a stressor (Perry and Capaldo 2011) and subsequent effects on hematocrit may account for the lack of a significant relationship between hematocrit and handling time in the species examined here. However, caution must be exercised because the relative contributions of splenic contractions and fluid shifts are highly context- and taxa-specific (reviewed in Gallaugher and Farrell (1998)).

Change-point analysis indicated that there was a shift from baseline in all species, with change-point times ranging from 2.0 to 6.8 min. This range illustrates a degree of interspecific variation that may correspond with performance and aerobic differences among species (Gallaugher and Farrell 1998; Wells et al. 2003; Crans et al. 2015), as is believed to be the case for mammals and birds (Carpenter 1975). Qualitatively, shifts in absolute hematocrit from baseline values were quite low, especially when compared with teleosts undergoing exhaustive exercise (Milligan and Wood 1987; Pearson and Stevens 1991; Wells et al. 2003; Suski et al. 2007). The abrupt shift in hematocrit and the corresponding absence of a linear relationship with time likely stem from the rapid actions of catecholamines released following acute stress. Here, catecholamines can cause a rapid increase in hematocrit through a release of additional erythrocytes from the spleen (Soivio and Oikari 1976; Yamamoto et al. 1985; Pearson and Stevens 1991; Rothwell 2005; Caldwell et al. 2006; reviewed in Randall and Perry (1992) and Perry and Capaldo (2011)). Over more extended durations, erythrocyte swelling can also occur via adrenergic mediated

Fig. 2. Relationship between blood glucose concentration (mM; equal to mmol·L^{-1}) and handling time (min) for six freshwater teleost species. The time at which blood [glucose] diverged from baseline status (i.e., the change point) is indicated by the vertical dotted line. In these species, regression lines were examined before (solid circles; N = 12–56) and after (open circles; N = 12–14) the change-point value, but were not significant. Where no change point was detected, a single regression line was fitted to the entire data set (solid circles, N = 39–66). The relationship between blood [glucose] and handling time was significant for (A) largemouth bass (*Micropterus salmoides*; [glucose] = 2.48 + (0.15 × time) – (2.14 × 10^{-3} × length); $p < 0.001$; $r^2 = 0.372$), (C) rock bass (*Ambloplites rupestris*; [glucose] = 1.36 + (0.11 × time) – (7.51 × 10^{-3} × length); $p = 0.015$; $r^2 = 0.134$), (D) bluegill (*Lepomis macrochirus*; [glucose] = 0.348 + (0.02 × time) – (4.85 × 10^{-5} × length); $p < 0.001$; $r^2 = 0.411$), and (F) northern pike (*Esox lucius*; [glucose] = 4.496 + (0.11 × time) – (3.64 × 10^{-3} × length); $p < 0.001$; $r^2 = 0.417$), but not for (B) smallmouth bass (*Micropterus dolomieu*) and (E) pumpkinseed (*Lepomis gibbosus*).

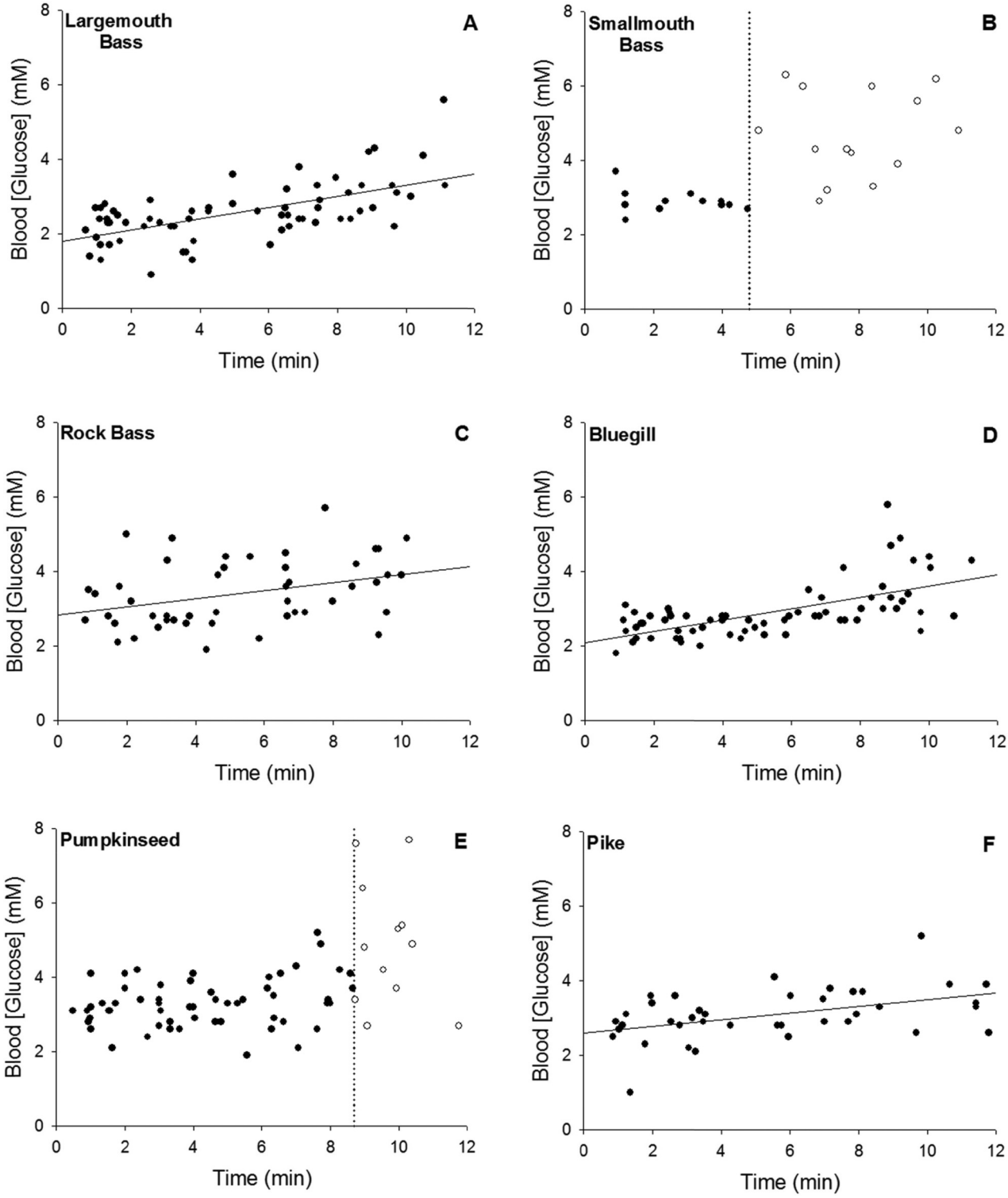

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Fig. 3. Relationship between blood glucose (mM; equal to mmol·L^{-1}) and plasma cortisol (ng·mL^{-1}) concentrations in five species of wild teleost fishes. Significant relationships were found in (A) largemouth bass (*Micropterus salmoides*; [glucose] = 2.26 + (0.02 × [cortisol]); $p < 0.001$; $r^2 = 0.247$; $N = 57$), (D) bluegill (*Lepomis macrochirus*; [glucose] = 2.55 + (0.01 × [cortisol]); $p < 0.001$; $r^2 = 0.403$; $N = 53$), (E) pumpkinseed (*Lepomis gibbosus*; [glucose] = 3.10 + (0.08 × [cortisol]); $p < 0.001$; $r^2 = 0.314$; $N = 45$), and (F) northern pike (*Esox lucius*; [glucose] = 2.89 + (4.25 × 10^{-3} × [cortisol]); $p = 0.017$; $r^2 = 0.139$; $N = 34$). No relationship was apparent in (C) rock bass (*Ambloplites rupestris*). No data were reported for plasma [cortisol] in (B) smallmouth bass (*Micropterus dolomieu*) so the graph is omitted. All plots were fitted with a simple linear regression.

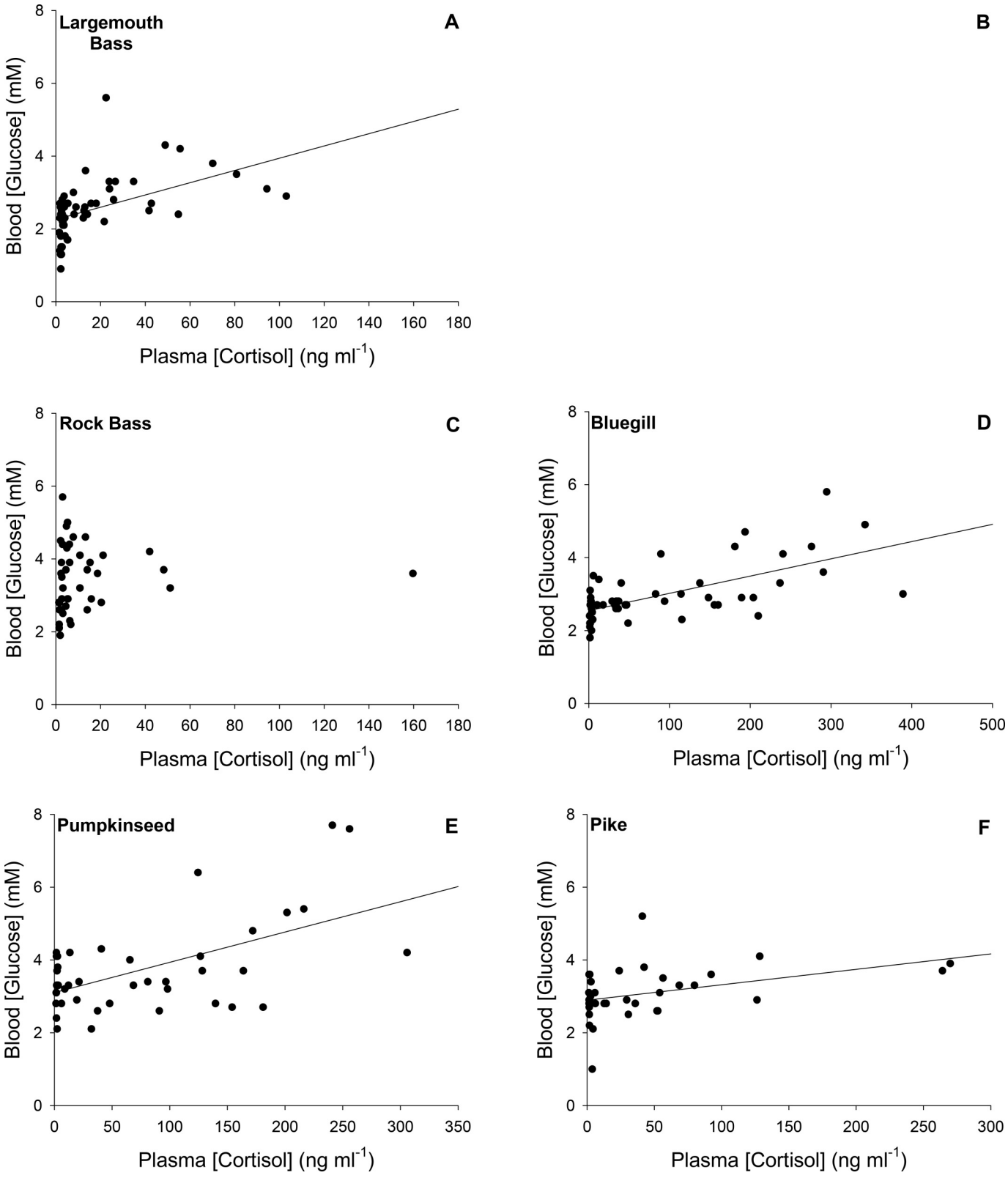

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Fig. 4. Relationship between blood lactate concentration (mM; equal to mmol·L^{-1}) and handling time (min) for six freshwater teleost species. The time at which blood [lactate] diverged from the baseline (i.e., the change point) is indicated by the vertical dotted line. Regression lines were fitted before (solid circles; N = 8–19) and after (open circles; N = 17–52) the change-point value. Significant relationships between blood [lactate] and time prior to the change point were found in (A) largemouth bass (*Micropterus salmoides*; [lactate] = 1.82 + (8.50 × time) – (0.01 × length); p = 0.031; r^2 = 0.227), (B) smallmouth bass (*Micropterus dolomieu*; [lactate] = –0.70 + (0.61 × time) + (2.50 × 10^{-3} × length); p = 0.035; r^2 = 0.474), (D) bluegill (*Lepomis macrochirus*; [lactate] = –0.08 + (0.62 × time) – (9.17 × 10^{-4} × length); p = 0.007; r^2 = 0.298), (E) pumpkinseed (*Lepomis gibbosus*; [lactate] = 1.44 + (0.48 × time) – (5.57 × 10^{-3} × length); p = 0.032; r^2 = 0.246), and (F) northern pike (*Esox lucius*; [lactate] = 2.47 + (0.85 × time) – (3.82 × 10^{-3} × length); p = 0.031; r^2 = 0.168), but not in (C) rock bass (*Ambloplites rupestris*). After the change point, this relationship was significant for (A) largemouth bass ([lactate] = 2.81 + (0.41 × time) – (3.40 × 10^{-3} × length); p < 0.001; r^2 = 0.367), (C) rock bass ([lactate] = 3.31 + (0.24 × time) – (6.14 × 10^{-3} × length); p = 0.008; r^2 = 0.178), (D) bluegill ([lactate] = 2.14 + (0.12 × time) – (2.72 × 10^{-3} × length); p = 0.015; r^2 = 0.111), and (F) northern pike ([lactate] = 5.84 + (0.33 × time) – (0.01 × length); p < 0.001; r^2 = 0.479), but not for (B) smallmouth bass and (E) pumpkinseed.

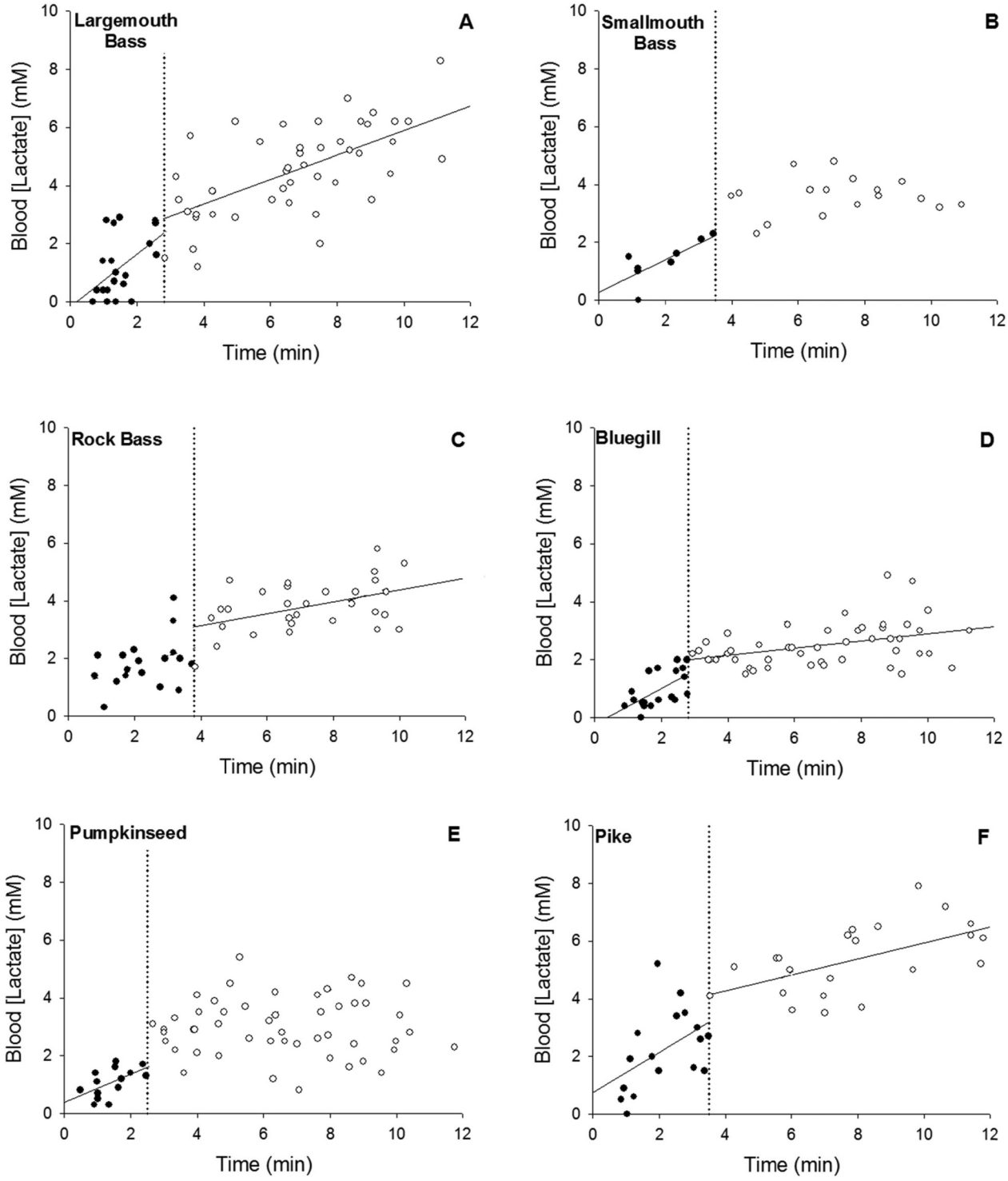

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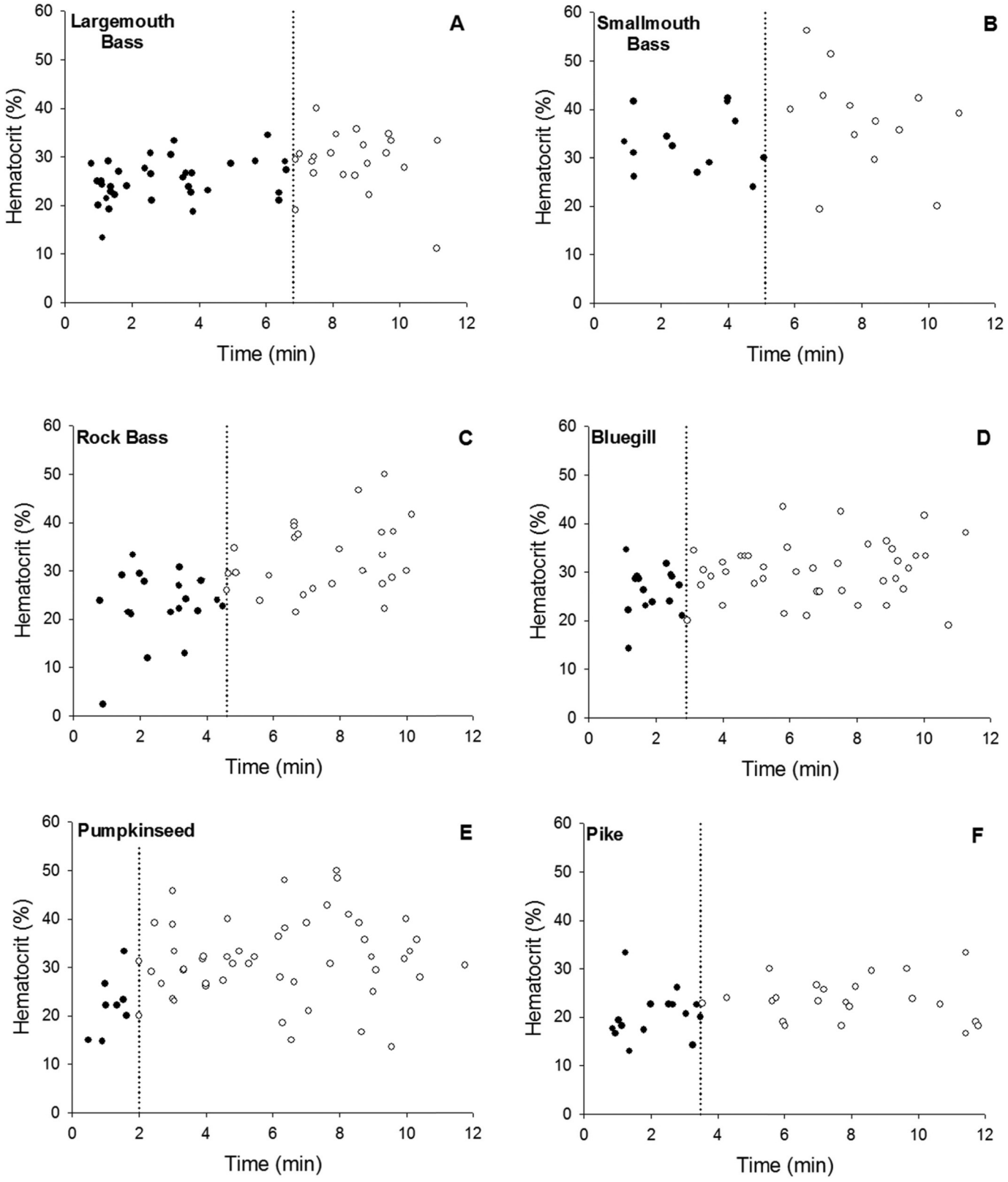
Fig. 5. Relationship between hematocrit (%) and handling time (min) for six freshwater teleost species: (A) largemouth bass (*Micropterus salmoides*); (B) smallmouth bass (*Micropterus dolomieu*); (C) rock bass (*Ambloplites rupestris*); (D) bluegill (*Lepomis macrochirus*); (E) pumpkinseed (*Lepomis gibbosus*); and (F) northern pike (*Esox lucius*). The time at which plasma cortisol diverged from the baseline (i.e., the change point) is indicated by the vertical dotted lines. Regression lines were examined before (solid circles; N = 8–34) and after (open circles; N = 13–50) the change-point value but were not significant and therefore are not shown.



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Na^+/H^+ exchange (Caldwell et al. 2006; reviewed in Randall and Perry (1992) and Perry and Capaldo (2011)). Taking into account the change-point analyses, it is reasonable to suggest a 2 min cutoff time for capturing "baseline" hematocrit, although the challenge of collecting a true baseline value for a variable such as haematocrit that is influenced by rapid adrenergic responses should not be overlooked. However, intraspecific variation in the change-point values was relatively large and, as such, a 2 min sampling window would present a reasonable time frame for allowing comparisons among species.

General recommendations and conclusions

This study presented a characterization of the timing and magnitude of blood physiology changes caused by an acute stressor over a fine temporal scale. In particular, Romero and Reid's (2005) 3 min rule for collecting a blood [glucocorticoid] sample that is representative of baseline conditions was evaluated, as was the applicability of this rule to other blood metrics. We demonstrated that both blood [glucose] and plasma [cortisol] remained relatively stable within a 3 min sampling window. In contrast, blood [lactate] and hematocrit were more sensitive to sampling time. Thus, the goals of the researcher will determine the appropriate sampling duration. Indeed, our findings do not take into account other blood parameters that can change on the order of seconds (e.g., catecholamine levels), and thus, the context and the biological dynamics of the metric of interest should be considered when establishing appropriate sampling durations. As in Romero and Reed (2005), we opt for a conservative approach in this timing suggestion whereby baseline blood metrics would be preserved across various intrinsic and extrinsic contexts. In contemporary biology, there is a growing movement to address physiological questions in a natural setting, especially in the context of the "ecology of stress" paradigm (Boonstra 2013) and the burgeoning field of "conservation physiology" (Cooke et al. 2013*b*). Ensuring that blood samples are representative of key physiological states (e.g., baseline) is important in addressing fundamental and applied questions related to teleost fish.

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