Diego Pereira Nogueira da Silva

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Effects of elevated temperatures and prolonged thermal stress on physiology and behaviour of the American bullfrog (*Lithobates catesbeianus*): correlations between stress physiology and animal personality

São Paulo

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Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre Ciências, na área de Fisiologia Geral.

Orientador: Fernando Ribeiro Gomes

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Prof(a). Dr(a).	Prof. Dr. Fernando Ribeiro Gomes Orientador

“After a billion years
The show is still here
Not a single one of your fathers died young
The handy travellers out of Africa
Little Lucy of the Afar

Gave birth to fantasy
To idolatry
To self-destructive weaponry
Enter the God of gaps
Deep within the past
Atavistic dread of the hunted

Enter Ionia, the cradle of thought
The architecture of understanding
The human lust to feel so exceptional
To rule the Earth

Hunger for shiny rocks
For giant mushroom clouds
The will to do just as you'd be done by
Enter history, the grand finale
Enter ratkind

Man, he took his time in the sun
Had a dream to understand
A single grain of sand
He gave birth to poetry
But one day'll cease to be
Greet the last light of the library”

Nightwish,
The Greatest Show on Earth

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Resumo Geral

Mudanças ambientais antropogênicas, em especial, mudanças climáticas, são um dos principais fatores associados com a grande crise de biodiversidade que vêm sido observada nas últimas décadas. Sabe-se que o aumento de temperatura global está associado com respostas de estresse tanto a nível hormonal quanto comportamental, observadas em diversas espécies na natureza, e que animais ectotermos se encontram especialmente vulneráveis. Recentemente, o estudo da personalidade animal (diferenças interindividuais consistentes de comportamento) e sua importância para entender como animais lidam com estressores e as implicações disso para a conservação vem ganhando bastante atenção. Neste trabalho foi investigada a existência de personalidade e como esta interage com a fisiologia do estresse em rãs-touro (*Lithobates catesbeianus*) quando elas são submetidas a temperaturas elevadas por períodos prolongados. Para cada animal, foram medidas a exploração e boldness múltiplas vezes antes e depois dos animais serem submetidos às temperaturas elevadas e também medimos as concentrações de corticosterona (CORT) e testosterona (T) ao longo do experimento. A temperatura elevada se mostrou um potente estressor para a rã-touro. Animais submetidos ao estressor tiveram maiores concentrações de CORT e menores concentrações de T durante toda a duração do experimento (~7 semanas). Além disso, indivíduos foram consistentes tanto para medidas de boldness quanto para exploração, havendo existência de personalidade. Porém, não foi encontrada uma síndrome comportamental entre esses dois eixos comportamentais. A personalidade se mostrou responsiva ao aumento de temperatura, diminuindo a consistência a longo termo para as variáveis exploratórias e aumentando a consistência de boldness. Finalmente, animais com maiores concentrações de CORT e menores concentrações de T exploraram seu ambiente mais rápido e se expuseram menos a riscos (mais tímidos), e animais mais tímidos também tiveram uma maior perda de condição corpórea ao longo do experimento. Esses resultados podem indicar que animais mais tímidos estariam mais vulneráveis às mudanças climáticas nessa espécie, com possíveis impactos negativos do aumento de temperatura à reprodução e sobrevivência desses indivíduos. Esta foi a primeira vez que correlações entre personalidade e diferenças proximais subjacentes foram demonstradas em uma espécie de anfíbio. Dado que anfíbios atualmente são o grupo de vertebrados mais ameçados pelas mudanças climáticas, estudos investigando interações da personalidade com mudanças ambientais se mostram promissores para auxiliar em programas de conservação e manejo de espécies ameaçadas.

General Abstract

Anthropogenic environmental change, particularly, climate change, is one of the most important factors associated with the great biodiversity crisis observed in the past few decades. It is known that rises in average global temperature are associated with stress responses at the hormonal and behavioural levels in several species in the wild, and that ectotherms are especially vulnerable to these changes. Recently, the study of animal personality (consistent interindividual differences in behaviour) e its importance to understand how animals cope with stressors, and the implications of this behavioural consistency for conservation has gained a lot of attention. In this work, I investigated the existence of personality differences and how they interact with stress physiology in the American bullfrog (*Lithobates catesbeianus*) when animals are exposed to elevated temperatures for long periods of time. For each animal, I measured exploration and boldness multiple times before and after exposure to high temperatures, and I also measured plasma concentrations of corticosterone (CORT) and testosterone (T) throughout the experiment. Elevated temperatures were shown to be a potent stressor for the bullfrogs. Animals exposed to thermal stress had higher levels of CORT and lower levels of T throughout the entire experiment (~7 weeks). Furthermore, individuals were consistent both in measures of boldness as well as exploration, indicating the existence of personality traits. However, there was no evidence for a boldness-exploration behavioural syndrome. Personality differences were responsive to increases in temperature, with long-term (across contexts) consistency being lowered for exploratory variables and increased for boldness. Finally, animals with higher plasma concentration of CORT and lower concentration of T explored their environment faster and were more risk-averse (shy), and shy individuals also had a larger loss of body condition throughout the experiment. These results might indicate that shyer animals are more vulnerable to climate change in this species, with possible negative impacts arising from elevated temperatures in reproduction and survival in a natural setting. This was the first time that differences in personality were correlated with possible underlying proximate mechanisms in a species of amphibian, which are notably understudied in personality literature. Given that amphibians are currently the most vulnerable vertebrate group in the context of climate change, studies investigating interactions between personality differences and environmental change are likely to be very promising in aiding programmes for management and conservation of endangered species.

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Lista de Abreviaturas

12h – Samples collected 12 hours after temperature increase
24d – Samples collected 24 hours after temperature increase
47d – Samples collected 47 hours after temperature increase
AD – Ambulatory distance
BI – Body index
CIDs – Consistent interindividual differences
CORT – Corticosterona
CT – Constant temperature group (28ºC)
EB – Eating behaviour
GCs – Glicocorticoides
HHA/I – Eixo hipotálamo-hipófise-adrenais/interrenais
HPA/I – Hypothalamus-pituitary-adrenal/interrenal axis
HPG – Hypothalamus-pituitary-gonadal axis
ID – Individual
IT – Increment temperature group (28ºC → 34ºC)
LM – Linear models
LMM – Linear mixed models
PC – Posture change
PCA – Principal component analysis
POLS – Pace-of-life syndrome
SVL – Snout-vent length
T – Testosterona
TC – Time on centre of the arena
WM – Wall movements

Índice

1. Introdução Geral

1.1. Mudanças climáticas e a resposta de estresse

Os impactos das mudanças climáticas antropogênicas na biodiversidade vêm crescendo nas últimas décadas, tornando-se um grande problema e tópico de debate e pesquisa acadêmica (Fig. 1.1). Atualmente, estima-se que a taxa de perda de diversidade em função da ação humana é a maior nos últimos 100 milhões de anos no chamado sexto grande evento de extinção em massa, ou extinção do holoceno/antropoceno (Turvey, 2009). Projeções de aumento da temperatura para as próximas décadas apontam diferentes cenários de maior ou menor risco ambiental com base em medidas tomadas para desacelerar a emissão de gases do efeito estufa (IPCC, 2014) (Fig. 1.2). Além disso, outras mudanças antropogênicas como alterações na disponibilidade de recursos, poluição, espécies invasoras e doenças emergentes atuam de forma sinergística, tornando o cenário ainda mais complexo (Russell et al., 2009; Clark et al., 2011; Strifling, 2011; Cabral et al., 2019). Nesse cenário de complexidade, apesar do crescente aumento no número de estudos sobre o impacto das mudanças climáticas na biodiversidade, ainda estamos longe de compreender completamente como diferentes animais respondem às mudanças ambientais (Jaeschke et al., 2014). Dessa forma, uma abordagem integrativa investigando diferentes aspectos de como os animais respondem a esses novos desafios ambientais se mostra extremamente importantes para que pesquisadores e instituições consigam propor medidas de conservação e manejo mais efetivas.

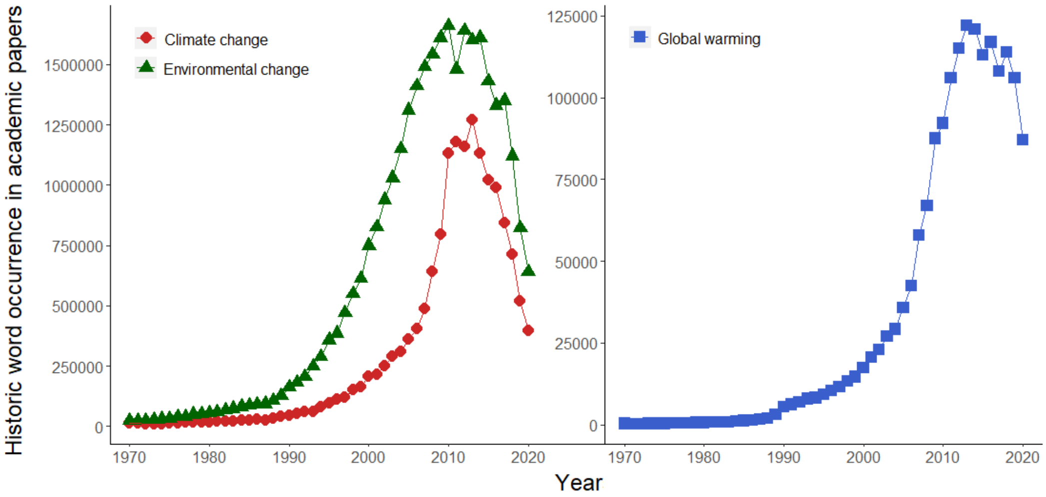

Figura 1.1: Número de ocorrências dos termos "climate change" (vermelho, ●), "environmental change" (verde, ▲), e "global warming" (azul, ■) em artigos acadêmicos nos últimos 50 anos. É possível notar um grande aumento de trabalhos investigando as mudanças ambientais antropogênicas nas últimas décadas. Com base em Strobel, 2018.

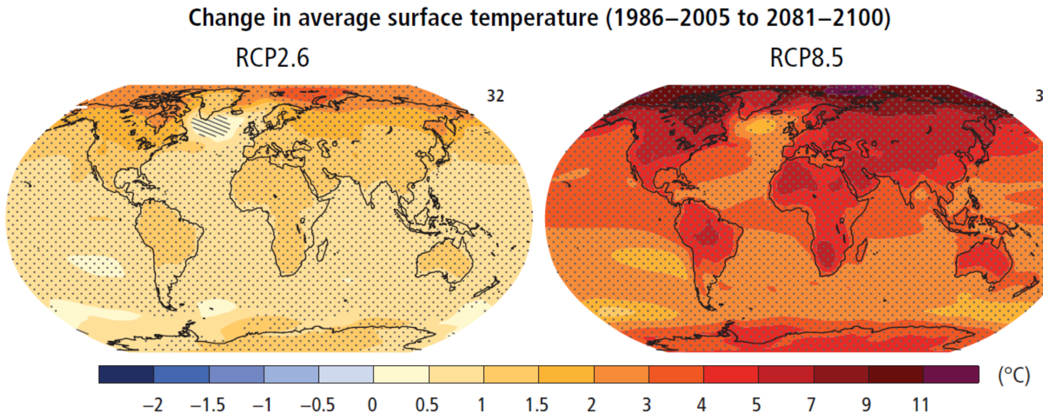

Figura 1.2: Mapas de projeção para o aumento da temperatura média superficial entre os anos de 2081-2100 considerando diferentes cenários de emissão de gases estufa. RCP2.6 representa um cenário com diminuição gradual de emissões de CO_2 ao longo do século 21, atingindo um patamar de emissão zero em 2100. RCP8.5 representa o pior cenário possível onde a emissão de gases estufa aumenta continuamente ao longo do século. Adaptado de “IPCC. (2014). Climate Change 2014: Synthesis Report”.

Essas mudanças ambientais, uma vez interpretadas pelos animais como estressoras, iniciam no organismo uma cascata de respostas neuroendócrinas que irão modular a forma como os animais lidam com esses estressores. Dentre estas, o aumento da ativação do eixo hipotálamo-hipófise-adrenais/interrenais (HHA/I) frente a um estímulo percebido como nocivo resulta em um aumento na concentração de glicocorticoides (GCs) na corrente sanguínea, estimulando a mobilização de reservas energéticas frente ao aumento de demanda energética associado ao estresse (Romero & Wingfield, 2016). Os GCs são hormônios esteroides que atuam principalmente através de receptores intracelulares, estando associados com regulação da transcrição gênica de diversos processos em diferentes tecidos do organismo (McEwen et al., 1986, porém, para revisões sobre receptores de membrana ver: Bosker, 2000 & Tasker et al., 2006). Essa ação dos GCs nos mais diferentes tecidos resulta em modulação de múltiplas funções do organismo. Por exemplo, é sabido que o aumento na concentração de GCs possui diversos efeitos sobre funções imunitárias (Dhabar, 2014) e reprodutivas (Geraghty & Kaufer, 2015). Ademais, em um cenário de estressores prolongados como ocorre frente a mudanças ambientais, o eixo HHA/I se encontra permamentemente estimulado, resultando em concentrações de GCs cronicamente elevadas e diversas fisiopatologias associadas, como supressão de funções imunitárias (Dhabar, 2000; Dhabar 2009) e inibição da reprodução em diversos pontos do eixo hipotálamo-hipófise-gônadas, levando à diminuição da secreção de esteroides gonadais (Tilbrook et al., 2000).

Nesse contexto, estudos investigando como aumentos de temperatura impactam a saúde de animais têm mostrado resultados interessantes. Por exemplo, em endotermos o estresse térmico associado a temperaturas elevadas fora de suas zonas de termoneutralidade, reduz a taxa metabólica desses animais (Boyles et al., 2011), alteram a dinâmica entre parasitas e hospedeiros, aumentando a suceptibilidade a infecção e o desenvolvimento e crescimento dos parasitas (Kelley, 1980; Morley & Lewis, 2014) e aumentam o estresse oxidativo (Zuo et al., 2000, Azad et al., 2010), impactando o desempenho de crescimento desses animais (Azad et al., 2010). Além disso, também foi demonstrado que o estresse térmico impacta negativamente diferentes variáveis reprodutivas em aves e mamíferos (Hill & Alliston, 1981; Rozenboim et al., 2007; Hansen, 2009; Rasooli et al., 2010). Quando analisamos animais ectotérmicos, resultados similares podem ser observados. Em diversos grupos diferentes de ectotermos, o estresse térmico também suprime variáveis imunes (Dittmar et al., 2014, Wojda, 2017; Huo et al., 2019, Lima et al., 2020) e reprodutivas (Narayan & Hero, 2014a; Porcelli et al., 2017; Fraser & Chan, 2019; Sales et al. 2021), gerando efeitos deletérios para o fitness desses animais. Ademais, os efeitos do estresse térmico se mostram ainda mais drásticos nos ectotermos, uma vez que o metabolismo e, consequentemente, desenvolvimento e reprodução desses animais depende diretamente da temperatura ambiental e de sua capacidade de termorregular comportamentalmente (Huey & Kingsolver, 2019; Kearney et al., 2009). Além disso, temperaturas muito elevadas, próximas de seu limite crítico máximo de tolerância térmica, podem rapidamente levar a morte do animal caso esse não seja capaz de selecionar microhabitats com temperaturas mais baixas (Lutterschmidt & Hutchinson, 1997). Por esses motivos, ectotermos em geral são considerados como sendo mais vulneráveis às mudanças climáticas do que endotermos (Aragón et al., 2010). Em conjunto, todos esses resultados vêm mostrando aos pesquisadores e instituições o importante papel da fisiologia da conservação em auxiliar o nosso entendimento a respeito dos impactos das mudanças ambientais sobre os animais (Wikelski & Cooke, 2006).

1.2. Comportamento, personalidade animal e estilos de enfretamentoo

Além das respostas fisiológicas, as respostas comportamentais também são importantes para auxiliar a sobrevivência dos animais frente a desafios ambientais (Romero & Wingfield, 2016). Foi demonstrada modulação pela temperatura em diversos comportamentos de ectotermos. Por exemplo, em espécies de escorpiões, peixes, anfíbios e répteis, foi observado um aumento de frequência em respostas antipredatórias mais ativas, como se expor a maiores riscos, maior agressividade, e maior proporção de respostas de fuga em comparação com respostas de freezing, em temperaturas mais altas, o que estaria associado com um aumento da taxa metabólica (Schieffelin & de Queiroz, 1991; Weetman et al. 1998; Gomes et al. 2002; Carlson & Rowe, 2009). Porém, interessantemente, em neonatos da serpente *Storeria dekayi*, foi observada a relação inversa, com serpentes sendo mais passivas, aumentando a resposta de tanatose, em temperaturas mais altas. Ao contrário do que se esperaria, a tanatose em neonatos dessa espécie parece ser mais energeticamente custosa do que uma resposta de fuga, explicando essa diferença encontrada (Gerald, 2008). O comportamento alimentar também é afetado pela temperatura. Diversos estudos apontam para um aumento na expressão de comportamentos alimentares de animais ectotermos em temperaturas mais altas (Mathavan & Pandian, 1975; Harrison & Fewell, 1995; Volkoff & Rønnestad, 2020). Porém, também já foi demonstrado em diversas espécies de peixes que há uma diminuição da taxa de alimentação quando temperaturas atingem valores próximos dos seus limites de tolerância térmica (Volkoff & Rønnestad, 2020), o que provavelmente reflete um padrão geral em ectotermos associado a perda de função de variáveis fisiológicas em temperaturas muito elevadas (Huey & Stevenson, 1979). Além disso, o aumento de temperatura também pode alterar a busca dos animais por alimentos com diferentes qualidades nutricionais (Traniello et al., 1984; Lemoine et al. 2013). Em um contexto diferente, em *Apis melífera,* maiores temperaturas ao longo do desenvolvimento larval estão associadas com um aumento na probabilidade de danças (comportamento associado com forrageamento na espécie), e com início do comportamento de forrageio mais cedo no desenvolvimento desses indivíduos, mostrando efeitos organizacionais da temperatura ao longo da ontogenia (Becher et al., 2009). Efeitos da temperatura também foram observados para diversos comportamentos reprodutivos como corte, acasalamento, territorialidade, transferência de esperma e oviposição. Em algumas espécies, temperaturas elevadas diminuíram comportamentos reprodutivos (Fasolo & Krebs, 2004) porém, em outras espécies, o contrário pode ser observado (Zamundio et al., 1995; Sentis et al., 2015) e em alguns casos machos e fêmeas apresentam respostas distintas (Katsuki & Miyatake, 2009). Finalmente, autores ressaltam a importância de destacar que efeitos da temperatura sobre o comportamento de ectotermos não são somente uma consequência do aumento metabólico, mas em muitos casos uma resposta integrada (Abram et al., 2017).

Esses resultados ilustram como a temperatura pode diretamente modular a expressão de diferentes comportamentos em animais a nível populacional. Porém, um tópico que vem ganhando mais atenção recentemente na literatura etológica é o estudo da personalidade e suas implicações para áreas como ecologia, evolução e conservação (Réale et al., 2007; de Azevedo & Young, 2021). Personalidade, também chamada de temperamento por alguns autores, é definida como diferenças interindividuais consistentes (CIDs, do inglês – *Consistent Interindividual Differences*) de comportamento, sendo essas diferenças consistentes ao longo do tempo e em diferentes contextos ecológicos (Réale et al., 2007). Notadamente, quando estudos são feitos tomando como base a média populacional, é possível que haja a perda de variação biológica de interesse e relevância ecológica: diferenças individuais na maneira como animais lidam com desafios ambientais. Diversos estudos vêm mostrando a importância dessas CIDs em diferentes contextos ecológicos. Por exemplo, diferenças de personalidade são importantes no processo de invasão, com animais ditos proativos (i.e.: animais que exploram seu ambiente mais rápido, se expõem mais a riscos, são mais agressivos e possuem maior rigidez comportamental) sendo mais invasivos em comparação com animais ditos reativos (Gruber et al., 2017a; Gruber et al., 2017b; Gruber et al., 2018). Similarmente, também foi demonstrada uma correlação entre personalidade e sucesso reprodutivo, com animais com características proativas apresentando maior sucesso reprodutivo (Ariyomo & Watt, 2012; Monceau et al., 2017). Em *Parus major*, por outro lado, casais formados por pais mais proativos e mães mais reativas tiveram maior sucesso reprodutivo, ilustrando a importância dessas duas personalidades coexistirem nessa espécie (Both et al., 2005). Embora essas CIDs existam, isso não significa que os animais não possam apresentar respostas plásticas frente a mudanças de contexto. De fato, foi demonstrado que aumentos de temperatura podem modular a personalidade dos animais (Biro et al., 2010; Briffa et al., 2013; Gribben et al., 2013; Zhao & Feng, 2015), porém, em diversos casos, é notada a presença de diferenças interindividuais nas normas de reação às mudanças de temperatura, adicionando mais uma camada de complexidade ainda muito pouco estudada na literatura. Por fim, dois estudos em peixes demonstraram que a temperatura preferencial de indivíduos mais proativos era mais elevada, o que poderia ter implicações para a dinâmica populacional dessas espécie frente a aumentos de temperatura média anual (Rey et al., 2015, Cerqueira et al., 2016).

Apesar do aumento de estudos investigando personalidade animal nas últimas décadas, os mecanismos proximais (genéticos, epigenéticos, fisiológicos) que geram essas CIDs ainda são pouco conhecidos e a maior parte dos estudos está restrita a aves e mamíferos (Coppens et al., 2010; Trillmich et al., 2018). Porém, dentro dessa literatura, outro conceito que surge é o de estilos de enfretamento (em inglês: *coping styles)*, definido como um conjunto coerente e consistente de respostas comportamentais e fisiológicas a um estímulo estressor. Foi demonstrado que animais ditos proativos também apresentam menores valores basais de GCs e uma menor reatividade do eixo HHA/I a estímulos estressores, além de também apresentarem maior reatividade catecolaminérgica, menor reatividade colinérgica e maiores concentrações de testosterona (T) em comparação com animais reativos (Koolhaas et al., 1999). Em função dessas diferenças comportamentais e fisiológicas, acredita-se que, no geral, indivíduos ditos reativos estariam menos vulneráveis a mudanças ambientais, uma vez que a maior amplitude de resposta fisiológica de estresse, combinada com a maior flexibilidade comportamental desses animais, permitiria com que eles lidassem com alterações em seu ambiente de forma mais eficiente (Cockrem, 2013). Porém, de acordo com a literatura do estresse, sabe-se que manter concentrações de GCs elevadas por períodos prolongados impacta negativamente a saúde e sobrevivência dos animais, além de também dificultar a capacidade desses animais de responderem a estimulos estressores secundários (Romero et al., 2009). De fato, foi demonstrado que iguanas marinhas nutricionalmente estressadas após o fenômeno do El Niño, animais que possuíam retroalimentação negativa do eixo HHI mais fraca apresentaram maior mortalidade (Romero, 2012). Interessantemente, um estudo recente em *Parus major* demonstrou que aves com personalidade mais reativa possuíam maiores concentrações plasmáticas basais e pós estresse de GCs, além de uma retroalimentação negativa do eixo HHA mais fraca (Baugh et al., 2017), indicando que esses animais poderiam estar mais vulneráveis em um cenário de estressores crônicos em função da ação humana. Dessa forma, todos esses resultados vêm mostrando a importância de se estudar personalidade animal e como esses conhecimentos podem ser aplicados para auxiliar em medidas de conservação (de Azevedo & Young, 2021).

1.3. Por que estudar anfíbios?

Com o aumento das mudanças ambientais antropogênicas, os anfíbios são hoje o clado de vertebrados em maior vulnerabilidade e risco de extinção (Fig 1.3, IUCN, 2021). Ademais, as mudanças nos regimes de precipitação que acompanham as mudanças de temperatura seriam mais impactante para anfíbios, que dependem inteiramente da água para sua reprodução e sobrevivência de indivíduos no estágio larval (Blaustein et al., 2010). Experimentos investigando os efeitos das temperaturas elevadas demonstraram que, em *Rhinella marina*, repetidos choques térmicos aumentaram a concentração de metabólitos urinários de corticosterona (CORT) de maneira crônica e também afetaram a capacidade dos animais de responderem a um estressor secundário (Narayan & Hero, 2014b). Em um experimento similar, a exposição a um estressor térmico por 30 minutos aumentou a concentração de CORT, diminuiu a concentração de T e diminuiu o desempenho locomotor dos animais (Narayan Hero, 2014a). Efeitos no comportamento também podem ser observados. Em *Rana aurora*, a capacidade dos animais de se movimentar por áreas abertas em um ambiente degradado foi diminuída em situações de maior temperatura ou menor umidade (Chan-McLeod, 2003) e em *Notophthalmus viridescens* animais passaram mais tempo em refúgios em altas temperaturas e baixa precipitação (Roe & Grayson, 2008). Alterações no comportamento reprodutivo também foram observadas, com diversas espécies apresentando uma alteração de sua fenologia, antecipando sua época reprodutiva em função de mudanças climáticas (Chadwick et al., 2006; Kusano & Inoue, 2008).

Tratando-se de personalidade, por possuírem desenvolvimento indireto, com um estágio larval que ocupa nichos completamente diferentes dos adultos, anfíbios constituem um modelo ideal para se estudar a manutenção das CIDs ao longo do desenvolvimento. Apesar disso, estudos de personalidade em anfíbios ainda são muito escassos em comparação com outros grupos de vertebrados (Kelleher et al., 2018). Embora nenhum outro trabalho tenha sido feito investigando a importância da personalidade em anfíbios em um contexto de mudanças climáticas, trabalhos em outros contextos demonstraram resultados interessantes e de potencial importância para a conservação de anfíbios. Por exemplo, em *Rhinella marina*, uma espécie invasora de grande relevância ecológica, foi demonstrado que animais mais proativos são mais dispersivos, estando associados com a expansão da frente de invasão (Gruber et al., 2017a; Gruber et al., 2017b; Gruber et al., 2018). Em outro estudo, com salamandras *Plethodon cinereus* em um ambiente fragmentado, foram demonstradas diferenças de personalidade em função da idade das manchas florestais, com animais sendo mais ativos em manchas mais antigas (Cosentino & Droney, 2016). Embora os motivos para essa diferença não estejam claros, esse estudo ilustra a importância da personalidade em um contexto de degradação ambiental por ação humana. Em *Lithobates sylvaticus*, foi demonstrado que indivíduos com comportamento mais proativo também tinham menor carga parasitária (Koprivnikar et al., 2012). Dados os efeitos sinergísticos entre mudanças climáticas e doenças emergentes (Blaustein et al., 2010), nota-se a importância de mais estudos investigarem a relação entre personalidade e risco de doenças.

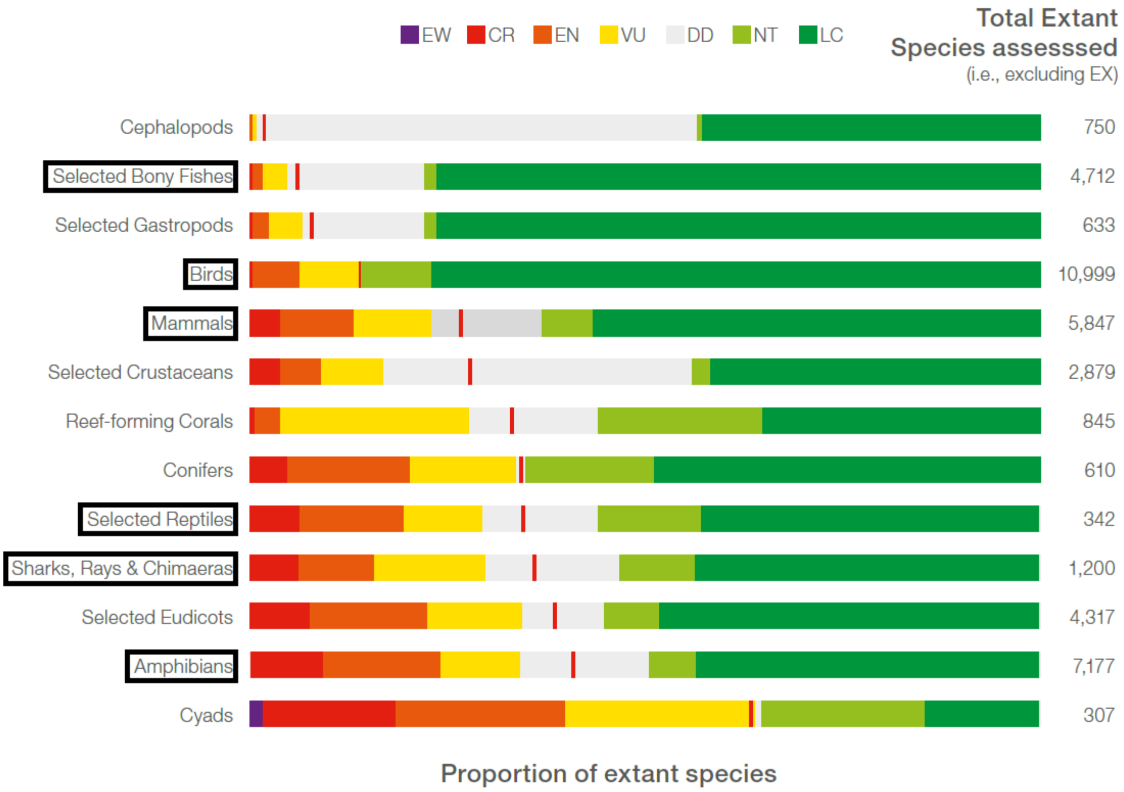

Figura 1.3: Proporção de espécies extantes em risco de extinção para diversos táxons. Táxons em destaque representam diferentes classes de vertebrados. Estima-se que cerca de 41% das espécies de anfíbios estejam ameaçadas. EW = Extinto na natureza; CR = Criticamente em perigo; EN = Em perigo; VU = Vulnerável; DD = Dados deficientes; NT = Quase ameaçada; LC = Pouco preocupante. Adaptado de IUCN, 2021.

Além disso, é notável o fato de nenhum outro trabalho ter investigado diretamente possíveis mecanismos proximais associados com a personalidade em anfíbios. Entretanto, é possível destacar alguns trabalhos que indiretamente analisaram potenciais mecanismos geradores de personalidade. Por exemplo, dois estudos identificaram diferenças de personalidade (Videlier et al., 2015) ou síndromes comportamentais (Aragón, 2011) em função do sexo. Dado que anfíbios apresentam determinação sexual genética, esses resultados poderiam ser explicados através de heranças ligadas aos cromossomos sexuais. Uma outra possibilidade (embora esta não seja mutuamente exclusiva da hipótese genética) seria a de que diferenças hormonais ou mesmo neuroanatômicas entre os diferentes sexos estariam por detrás dessas CIDs. Um outro estudo no sapo *Pseudophryne corroboree* demonstrou uma correlação entre diferenças de personalidade e o tamanho dos animais, com animais maiores explorando o ambiente mais rápido (Kelleher et al., 2017), dando suporte para o modelo de personalidade dependente de estado, onde estado seria uma característica subjacente do animal (e.g.: morfológica, fisiológica, neurobiológica) que afeta os custos e benefícios de suas respostas comportamentais (Dingemanse & Wolf, 2010). Finalmente, um estudo demonstrou a importância de experiência prévia ao longo da ontogenia para a geração de personalidade. Girinos criados sozinhos e sem pistas predatórias demonstraram ausência de personalidade. Porém, na presença de pistas predatórias, animais apresentaram comportamentos consistentes, e na presença tanto de pistas predatórias quanto de conspecíficos, foi observada a manifestação de síndromes comportamentais (Urszán et al., 2015). Esses resultados parecem apontar que, para essa espécie, a interação entre genótipo e ambiente é necessária para a manifestação de CIDs. Todos esses resultados mostram como personalidade em anfíbios é uma área promissora, embora pouco estudada. Conforme mais espécies forem sendo estudadas em diferentes contextos ecológicos, conhecimento sobre personalidade de anfíbios deve se tornar uma potencial ferramente em auxiliar na conservação desse grupo (Kelleher et al., 2018).

1.4. A rã-touro (Lithobates catesbeianus)

A rã-touro (Fig 1.4) é uma espécie originalmente norte-americana a qual foi introduzida em diversos outros países e atualmente é invasiva em todos os continentes com exceção da Antártica. Indivíduos de *L. catesbeianus* direta e indiretamente impactam a fauna de anuros nativos (e.g.: através de predação, competição e transmissão de doenças) (Snow & Witmer, 2010; Miaud et al., 2016). Particularmente na América do Sul, estudos prevém uma interação das mudanças climáticas com o processo de invasão, apontando para uma vulnerabilidade atual da Mata Atlântica a essa espécie em um contexto de mudanças climáticas, tal qual uma vulnerabilidade futura de regiões da América do Sul onde essa espécie ainda não se encontra (Nori et al., 2011). Dada a importância da personalidade no processo de invasão de *Rhinella marina* na Austrália (Gruber et al., 2017a; Gruber et al., 2017b; Gruber et al., 2018), estudos de personalidade em *L. catesbeianus* se mostram promissores para potenciais medidas de conservação e manejo futuras. Além disso, a rã-touro é a segunda espécie de anuro melhor estudada (de acordo com resultados de pesquisas no Google Scholar para espécies de anuro modelo), estando atrás apenas de *Xenopus laevis* em números de artigos publicados para a espécie. Dentre esses artigos, é notável a descrição de toda a neuroanatomia da espécie

(Wilczynski, 1981; Wilczynski & Northcutt, 1983). Por esses motivo, a rã-touro constitui um modelo anuro ideal para se estudar (1) personalidade em um contexto de mudanças ambientais e (2) os mecanismos proximais (fisiológicos e neuroanatômicos) da personalidade em anuros. Ao presente momento, personalidade na rã-touro havia sido estudada somente em girinos da espécie (Smith & Doupnik, 2005; Carlson & Langkilde, 2013), este sendo o primeiro trabalho a avaliar personalidade de adultos. Dessa forma, o presente trabalho constitui um passo inicial para estudos futuros investigando a personalidade nesta espécie.

Figura 1.4: Macho adulto de rã-touro (*Lithobates catesbeianus*). Autoria: Diego Pereira Nogueira da Silva.

1.5. Objetivos

O objetivo geral deste trabalho foi avaliar como a personalidade animal e a fisiologia do estresse interagem quando animais são expostos a temperaturas elevadas por períodos prolongados, pensando em um contexto de mudanças climáticas com aumento da temperatura média global.

Os objetivos específicos foram:

(1) Avaliar a resposta de estresse em *L. catesbeainus* quando animais são expostos continuamente a temperaturas elevadas;

(2) Identificar diferenças de personalidade e síndromes comportamentais em adultos de *L. catesbeianus;*

(3) Avaliar como o aumento da temperatura interage com a personalidade;

(4) Avaliar possíveis correlações entre personalidade e fisiologia do estresse em *L. catesbeianus*, buscando entender possíveis mecanismos proximais que geram a personalidade;

(5) Identificar se indivíduos com determinadas personalidades estariam mais vulneráveis ao aumento da temperatura em um cenário de aquecimento global.

As hipóteses foram:

(1) Frente à exposição a temperaturas cronicamente elevadas, adultos de rã-touro irão apresentar uma resposta de estresse;

(2) Adultos de rã-touro irão ter diferenças de personalidade;

(3) As diferenças de personalidade não serão afetadas pela temperatura;

(4) A personalidade será correlacionada com a atividade dos eixos HHI e HHG antes e depois dos animais serem expostos a altas temperaturas.

Para testar essas hipóteses: **(1)** os animais foram divididos em dois grupos, um grupo mantido em temperatura constante (28°C) ao longo de todo o experimento e outro grupo onde a temperatura foi aumentada (28 → 34°C) na metade do experimento. Foram coletadas amostras de sangue para dosagem hormonal antes e depois do aumento da temperatura para avaliar a resposta de estresse. **(2)** Cada indivíduo foi submetido aos testes comportamentais múltiplas vezes para avaliar a a existência de CIDs. **(3)** Os animais foram submetidos aos testes comportamentais antes e depois do aumento da temperatura no grupo experimental, permitindo assim avaliar se houve mudanças de personalidade ao longo do experimento. **(4)** Serão correlacionadas as medidas hormonais (CORT e T) e de personalidade de acordo com os pontos 1 e 2 acima.

Caso as hipóteses se corroborem, espera-se que: **(1)** animais estressados terão maiores concentrações plasmáticas de CORT e menores de T ao longo de todo o experimento; **(2)** os comportamentos analisados terão valores significativos de repetibilidade; **(3)** as estimativas individuais de personalidade antes e depois do aumento da temperatura serão correlacionadas; **(4)** animais com uma personalidade mais proativa terão menores concentrações basais e pós-estresse de CORT e maiores concentrações basais e pós-estresse de T.

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2. Elevated temperature effects on animal personality: hormonal stress response underlying behavioural differences in the American bullfrog

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2.1. ABSTRACT

Interest in consistent interindividual differences in behaviour, or animal personalities, have grown over the years in behavioural ecology literature. Differences in personality have been shown to be associated with how animals handle environmental challenges and their accompanied stress response in what is termed coping styles. It is expected, based on literature for birds and mammals, that reactive animals (shy, slow-explorers) should have a more pronounced stress response which could aid these animals in responding to environmental challenges. Here, we were interested in evaluating how personality might be correlated with the thermal stress response to elevated temperatures in an ectotherm, the American bullfrog, *Lithobates catesbeianus*. Animals were divided into two groups, the control was kept constantly at 28°C and the experimental group was initially kept at 28°C and later transferred to 34°C. We took plasma samples and measured the exploratory and risk-taking behaviour of animals from both groups before and after temperature increase. We have found that elevated temperatures significantly increased levels of plasma corticosterone (CORT) and decreased levels of plasma testosterone (T) for up to seven weeks and that animals did not acclimate to the changes in temperature for the duration of the experiment. Boldness and exploration were significantly repeatable, but there was no evidence of a behavioural syndrome. Thermal stress significantly affected animal personality, decreasing behavioural consistency on the long-term for exploration and increasing consistency in boldness. We found that individuals with higher plasma CORT and lower plasma T were shyer and explored their environment faster, and shyer individuals also lost more body condition throughout the experiment. Our results differ from what is usually reported on coping styles literature, suggesting that bolder animals should be less risk than shyer animals in the face of climate change.

Key-words: Amphibia, behavioural endocrinology, boldness, climate change, coping styles, corticosterone, ecophysiology, exploration, testosterone, thermal stress

2.2. INTRODUCTION

The study of animal personality, or consistent interindividual differences in behaviour, has gained a lot of attention in recent years. Personality differences have been observed in different species, ranging from cnidarians (Briffa & Greenaway, 2011) to a wide range of vertebrates (Gosling, 2001; Cockrem, 2007; Toms et al., 2010; Waters et al., 2017; Kelleher et al., 2018), therefore being widespread across different phylogenetic groups regardless of the behavioural complexity system. Studies have explored personality under different ecological contexts. For instance, personality has been demonstrated to be important in the process of biological invasion of several species, with animals with proactive characteristics (i.e.: animals which are consistently bolder, faster explorers, more aggressive and less behaviourally flexible) being more dispersive than reactive animals (Pintor et al., 2010; Gruber et al., 2018). This finding is likely to be related to a more general phenomenon of personality dependent dispersal that has been demonstrated in several species (Cote et al., 2010b). Dispersal is important in the context of environmental change because it reflects different coping strategies to environmental challenges, i.e., whether the animal will stay in the disturbed environment or leave (Astheimer et al., 1992; Denver, 1997; Romero & Wingfield, 2016). In a different context, personality has been related with thermal preference of fish. Notably, proactive animals consistently chose higher temperatures when exposed to a temperature gradient (Rey et al., 2015, Cerqueira et al., 2016), which is likely to be important in the context of climate change with increased average temperatures. Finally, in the red-backed salamander (*Plethodon cinereus*), personality differences were related with distribution across a fragmented landscape with high risk of desiccation, with individuals in older forests (i.e., in further stages of ecological succession) being more active (Cosentino et al., 2016). Together, these results emphasize the importance of personality in the response of animals to environmental change, having implications for management and conservation of vulnerable species (de Azevedo & Young, 2021)

Despite there being an extensive literature regarding the ecological aspects of personality, the underlying proximate mechanisms (i.e.: genetic, epigenetic, physiological, neurobiological) that give rise to this behavioural consistency are still notably understudied (Coppens et al., 2010; Trillmich et al., 2018). Specifically regarding endocrine underlying mechanisms, research has mostly focused on mammals and birds, with few studies on teleosts (Fürtbauer et al., 2015) and lizards (Kabelik et al., 2022). One concept that emerges in this literature is that of coping styles, which integrates consistency in both behavioural and physiological stress response variables (Koolhaas et al., 1999). The stress response is complex and involves several different neuroendocrine systems (Fink, 2016). However, the majority of research focuses on the increased activity of the hypothalamus-pituitary-adrenal/interrenal (HPA/I) axis which culminates in increased concentrations of circulating glucocorticoids (cortisol/corticosterone) (Chrousos & Gold, 1992; Nicolaides et al., 2015), enhancing metabolism and energy mobilization necessary for the animal to cope with the stressor (Sapolsky et al., 2000; Romero & Wingfield, 2016). Corticosterone (CORT) is a steroid hormone which is associated with widespread changes on transcription in target tissue and thus affects a wide range of phenotypical variables, including behaviour (McEwen et al., 1986; Beato & Klug, 2000). For instance, baseline increases in CORT levels outside of stressful situations are temporally correlated with daily and seasonal variation in activity, increasing immediately before and during periods of increased activity (Landys et al., 2006). Likewise, further elevations of CORT in response to stressors also impacts locomotor activity in a seemingly context dependent manner. For instance, in passerine birds, elevated CORT decreased activity if animals had *ad libitum* food, but increased activity when food access was restricted (Astheimer et al., 1992). In the coping styles framework, proactive animals are also found to have lower activity and reactivity of the HPA/I axis, having lower baseline and post-stress levels of CORT in comparison with reactive animals (Koolhaas et al., 1999).

The HPA/I axis also interacts with other neuroendocrine axes. Notably, the relationship with the hypothalamus-pituitary-gonadal (HPG) axis has been well studied. Repeated or continuous activation of the HPA/I axis has been shown to profoundly inhibit all levels of the HPG axis, resulting in lower circulating gonadal steroids, such as testosterone (T) and lower reproductive output (Moore & Jessop, 2003; Toufexis et al., 2014). Like CORT, T mechanism of action also involves transcription regulation in target tissue, modulating a wide range of variables as a function of reproduction (Beato & Klug, 2000). For instance, experimental increases of T in striped mice (*Rhabdomys pumilio*) increased boldness and reduced anxiety in these animals (Raynaud & Schradin, 2014). Likewise, in rats, increased T levels resulted in increased exploratory behaviour and motor activity (Zhang et al., 2011). Furthermore, proactive animals have been shown to have lower testosterone activity in comparison with reactive animals (Koolhaas et al., 1999). Besides these possible activational effects of both CORT and T, the endocrine modulation of behavioural consistency also involves organizational effects via permanent changes in protein expression in the brain during ontogeny (Moore, 1991; Rhen & Crews, 2002). Studies in rodents and birds have shown that administration of CORT and T during early life stages had potent effects on the manifestation of some personality traits and behavioural syndromes (i.e.: correlated suite of behaviours) in adults of those species (Partecke & Schwabi, 2008; Guenther et al., 2018). However, not all behavioural axes were affected by the experimental treatments, suggesting behavioural modularity and complexity in the relationship between endocrine and behavioural axes.

Anthropogenic environmental change is one of the biggest and most well studied causes of stress in wildlife animals (Daszak et al., 2001; Acevedo-Whitehouse & Duffus, 2009). Climate change in particular, characterized by elevation in global average temperature and changes in precipitation regimes has been extensively studied (Root et al., 2003; Blaustein et al., 2010; Fuller et al., 2010; IPCC, 2014; Lacetera, 2019; Soravia et al., 2021). Continuous temperature increase is known to elicit potent stress responses in both endotherms and ectotherms, impacting metabolism, growth, immunity and reproduction (Hill & Alliston, 1981; Hansen, 2009; Azad et al., 2010; Rasooli et al., 2010; Boyles et al., 2011; Dittmar et al., 2014; Morley & Lewis, 2014; Narayan & Hero, 2014a; Porcelli et al., 2017; Lima et al., 2020; Sales et al. 2021). In ectotherms, metabolism is a direct function of environmental temperature and their ability to behaviourally thermoregulate (Huey & Kingsolver, 2019; Kearney et al., 2009). Furthermore, elevated temperatures close to their thermal tolerance limits can quickly lead to death (Lutterschmidt & Hutchinson, 1997). For these reasons, ectotherms are considered to be at higher vulnerability when faced with climate change (Aragón et al., 2010). Additionally, temperature has also been shown to modulate several behaviours in ectotherms, such as antipredator (Weetman et al. 1998; Gomes et al. 2002; Gerald, 2008; Carlson & Rowe, 2009), eating (Mathavan & Pandian, 1975; Traniello et al., 1984; Volkoff & Rønnestad, 2020; Becher et al., 2009; Lemoine et al. 2013), and reproductive behaviours (Zamundio et al., 1995; Fasolo & Krebs, 2004; Katsuki & Miyatake, 2009; Sentis et al., 2015). Finally, personality differences can also be modulated by temperature (Biro et al., 2010; Briffa et al., 2013; Gribben et al., 2013; Zhao & Feng, 2015). In this context, personality research predicts that individuals with reactive personalities should be better equipped to deal with changes in environment, both due to their greater behavioural flexibility and due to their accentuated hormonal response (Cockrem, 2013; Geffroy et al., 2020). However, when stress remains constant, the reactive scope model also predicts that maintaining elevated levels of glucocorticoids for prolonged periods of time can be majorly detrimental to animal health and their capacity to respond to secondary stressors (Romero et al., 2009; Romero, 2012; DuRant et al., 2016). Therefore, the relationship between personality types and vulnerability to climate change should be complex and context dependent, and more research needs to be made investigating this relationship on the long term (i.e.: spanning months to years) and in different species.

Among vertebrate ectotherms, amphibians are especially vulnerable to climate change (Blaustein et al., 2010; IUCN, 2021). Past research has shown how elevated temperatures can impact the stress response in amphibians, increasing CORT, decreasing T, and also limiting their response to secondary stressors (Narayan & Hero, 2014a; Narayan & Hero, 2014b). Effects on behaviour have also been demonstrated, with high temperatures associated with desiccation risk limiting movement activity (Chan-McLeod, 2003) and increasing refuge use (Roe & Grayson, 2008). In personality literature, amphibians are still notably an understudied in comparison with other vertebrates, but existing research has shown interesting results, with implications for conservation and management (Kelleher et al., 2018). The American bullfrog (*Lithobates catesbeianus*) is a large ranid species native to North America. Currently, it has invaded most of the world, being found in nearly every continent and being directly and indirectly associated with the decline of native amphibian populations (Ficetola, 2007; Miaud et al., 2016). Furthermore, it constitutes the second most studied amphibian species (based on number of Google Scholar results for different anuran species), and, due to their success as an invasive species, one should expect bullfrogs to be highly adaptable and plastic. For all of these reasons, we consider *L. catesbeianus* to be a good species to investigate neuroendocrine e behavioural stress responses to environmental change in amphibians. Although the bullfrog is not threatened, we believe this study to be a first step towards evaluating how personality studies could help in management and conservation efforts of amphibians in face of climate change.

Here, we used bullfrogs bought from a local farm to investigate how animal personality and stress physiology interact when animals are continually exposed to elevated temperatures. We hypothesized that (1) continually elevated temperatures would elicit a stress response in this species; (2) individuals would have personality differences and behavioural syndromes; (3) personality differences would not be affected by temperature; and (4) personality differences would be explained by underlying differences in activity of the HPI and HPG axes before and after stress. To test these hypotheses, we submitted animals to temperatures close to their maximum voluntary temperature (Guevara-Molina et al., 2020) for up to seven weeks and we collected plasma samples and conducted behavioural assays before and after animals were exposed to high temperatures. If elevated temperatures are a stressor, we should expect to find an increase in plasma CORT and decrease in T concentrations. If animals have personality differences and behavioural syndromes, behaviours should be significantly repeatable and correlated with each other. And if personality differences are not affected by temperature, then personality estimates before and after thermal treatment should be correlated. Finally, if differences in stress physiology explain differences in personality, we expect that animals with proactive personalities (i.e.: bolder and faster explorers) would have lower baseline and post-stress plasma CORT and higher baseline and post-stress plasma T.

2.3. MATERIALS & METHODS

2.3.1. Studied animals and experimental conditions

Thirty adult male American bullfrogs (*L. catesbeianus*) were bought from a local farm in São Paulo, Brazil (Rã's World – 23°28'4.8"S, 46°42'7.2"W) and brought to laboratory conditions. Animals were obtained during their reproductive season (Medeiros et al., 2016), at the end of November 2018 and kept until March 2019. A timeline of the experiment can be seen on Fig. 2.1. All animals were kept at a constant photoperiod of 13:11 L:D, corresponding to the natural photoperiod these animals are exposed to during the summer and initially at 28±1°C inside climatic chambers (FITOTRON 011; Eletrolab). Animals were weighed and had their snout-vent length (SVL) measured up to the nearest 0.01g. Each bullfrog was individually housed inside opaque 20L plastic buckets (37cm height, 41cm superior diameter, 26cm inferior diameter) with ~4cm of aged tap water and a 15cm PVC tube (75mm diameter) placed diagonally inside the bucket for shelter. Water was changed at least once a week with aged tap water, depending on the observed turbidity. Animals were fed weekly with two pellets of carnivorous fish food (Guabi© Pirá Carnívoros) following the frog farm's instructions. For unknown reasons, 20 out of our 30 individuals did not display normal eating behaviour even when approached with food near their mouths and therefore we began force feeding these animals with two pellets of fish food after one month in captivity. We believe this might have been due to stress since 2 out of the 4 individuals that displayed normal eating behaviour in the experimental group (see below) stopped eating following temperature increase. This unintended difference in eating behaviour between different animals added an unintended factor into our experiment. However, this was factored into our analyses (see *Statistical Analyses*) and all animals were weighed weekly in order to monitor variation in body index (BI – taken as the residuals of a linear regression between mass as dependent variable and SVL as independent variable) throughout the experiment.

Animals were randomly divided into two groups of equal size (n = 15) and there was no initial difference in BI between the two groups (t = -0.169, p = 0.867). The control group (CT, constant temperature) was kept at a constant temperature of 28±1°C throughout the entire experiment which lasted 106 days, whereas the experimental group (IT, increment temperature) was kept at 28±1°C for 59 days and then at 34±1°C for the remainder of the experiment (Fig. 2.1). These temperatures were decided based on pilot experiments as well as a previous study which determined the bullfrog average preferred and maximum voluntary temperature to be 28ºC and 36ºC, respectively (Guevara-Molina et al., 2020). CT group had 6 individuals which displayed normal eating behaviour and 9 individuals were force fed, meanwhile, the IT group had 4 individuals with normal eating behaviour and 11 individuals which were force fed. Animals were given 15 days of habituation to laboratory conditions before beginning the experiments.

All experiments and use of biological material were approved by the ethics committee at University of Sao Paulo (CEUA nº 335/2018).

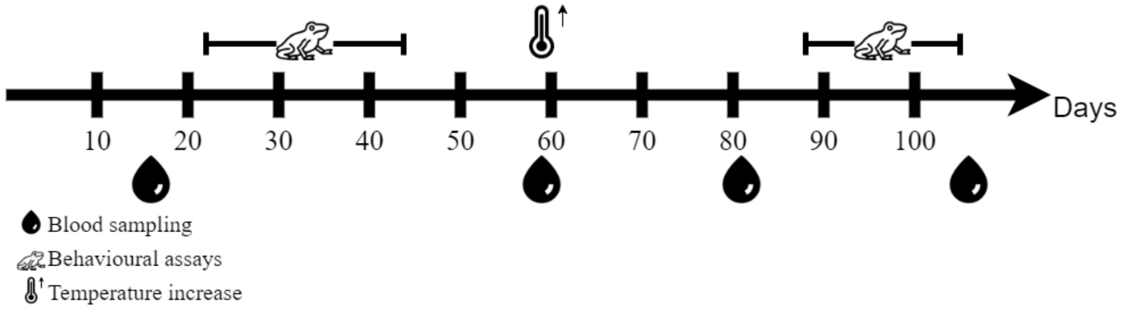

Figure 2.1: Timeline of the experiment. Experiment lasted 106 days from the day animals arrived at the laboratory up until the day of the last blood sample. Days of blood sampling, behavioural assays and temperature increase in the experimental group are marked.

2.3.2. Behavioural assays and variables

To test animal behaviour and personality, each bullfrog was placed in the centre of an 8-arm maze (arm dimensions: 30 x 15 x 20 cm; regular octagon side: 15 cm) made out of white polypropylene and covered with transparent acrylic lids. Animals were left to freely explore the maze for 30 minutes while the experimental arena was recorded with a camera placed directly above it. Between each trial, the arena was thoroughly cleaned with 70% ethyl alcohol in order to remove any odours or secretions left by the previous bullfrog. To calculate repeatability, each animal from CT and IT groups was tested three times before temperature was increased in the IT group, and again three times after (Fig. 2.1), for a total of six behavioural assays per individual. Each individual was given a week of rest between sequential trials. Furthermore, animals were given a 1-week minimum period of rest between blood sampling and behavioural assays to avoid possible behavioural interferences. One individual from the IT group died before the final trial and three videos were excluded from analyses due to animals escaping the maze during the 30 minutes trial and (n = 176).

All videos were manually analysed by the same author (DPS) for methodological consistency. We looked at a total of four different behaviours in our analyses. 1) Ambulatory distance (AD), corresponding to the total distance the animal travelled during the trial. AD is a direct measure of how much the animals explored their environment in terms of distance travelled. 2) Number of posture changes (PC), including head movements, lowering/lifting of the body, rotations, and extension/retraction of limbs. Since bullfrogs are animals that tend to stay in place for long periods of time, as well as sit-and-wait and wait predators, we believe PC can be a measure of how much they gather information from their environment while staying in place (e.g.: through visual, auditory or tactile stimuli), and thus also be associated in the process of decision making. 3) Number of movements against the walls of the maze (WM), including jumps against the wall and wall-hugging. WM could represent both attempts at escaping the arena which could be associated with fear or anxiety as is the case for rodents (Seibenhener & Wooten, 2015), but it could also represent a proxy for how much distance the animals would have covered had them be able to move freely. 4) Total amount of time the animals spent in the centre of the arena instead of the arms (TC), with an animal being considered in the centre of the arena if over half its body was in the centre. We only counted TC if the animals stayed still in the centre for at least 5 seconds, therefore controlling for a possible overinflation of TC due to differences in AD. Since staying still in the open could increase their risk of predation in a natural setting, TC is thought to be a measure of risk-taking behaviour (Yuen et al., 2017).

2.3.3. Blood sampling and hormonal analyses

Blood was collected from all individuals through cardiac puncture with heparinized 1mL syringes and 23Gx1/2” hypodermic needles. A total of 700-1000µL of blood was collected in each sample. Four samples were taken throughout the experiment: after 15 days of habituation to laboratory conditions, and three other times 12 hours, 24 days and 47 days after temperature was increased in the IT group (Fig 2.1) – samples were respectively denoted as baseline, 12h, 24d and 47d. All blood samples were collected 1 hour after the beginning of the dark phase and within 3 minutes of handling the animals in order to avoid possible hormone changes due to handling stress (Romero & Reed, 2005). Blood samples were kept in ice until processing. Blood was then centrifuged (3 min, 604 g) for plasma separation, and plasma was then stored at -80°C until hormone analyses.

Plasma was extracted according a previous study (Assis et al., 2015). In short, 3mL of diethyl ether was added to the plasma samples and then vortexed for 30 seconds and centrifuged for 9 minutes at 4°C and 1800 rpm. Samples were decanted at -80°C for 7 minutes and the liquid phase was transferred to a different tube and allowed to evaporate under a hood at room temperature. For hormonal assays, samples were resuspended in EIA buffer and corticosterone (CORT) and testosterone (T) were assayed with ELISA kits (Cayman Chemical) according to manufacturer’s instructions. Inter and intra-assay coefficients of variation were, respectively, 5.73 and 10.39 for CORT, and 10.5 and 16.77 for T. Sensitivity of the assays was 25.63 and 8.65 pg/ml for CORT and T, respectively.

2.3.4. Statistical analyses

Descriptive statistics for all hormone and behavioural variables can be found on Table 2.1. Variables were transformed to better fit the requirements of parametric tests (log10 for CORT, T; log10(x + 1) for AD, PC, and WM; square root for TC). We then tested for normality using the Shapiro-Wilk test.

We performed a Principal Component Analysis (PCA) on behavioural variables (AD, PC, WM, TC) in order to identify possible axes of variation in our data. PCA was performed with average values for each variable per individual, data was scaled and centred around zero prior to analysis. We then implemented a permutation-based significance test of the principal components and variables contributing to variation inside them using ‘PCAtest’ (Camargo, 2022). This method calculates significance in eigenvalues alone (ψ) as well as significance of eigenvalues in combination with the number of variables (φ).

To calculate adjusted repeatability, we used the 'rptR' package for Gaussian data (Nakagawa & Schielzeth, 2010). Individuals were computed as a random factor and time (before or after temperature increase), eating behaviour, and group were used as fixed factors. Since we were interested in how temperature treatment might affect personality, we also computed repeatability estimates separately for before and after experimental treatment to identify if individuals showed behavioural consistency in both instances. For this separate analysis, we used only eating behaviour and group as fixed factors. We then computed Pearson's correlation coefficients for personality estimates (taken as the mean value for each behavioural variable per individual) before and after experimental treatment for each group in order to identify long-term consistency over different contexts. We also computed Spearman's rank correlations coefficients for the rank order of each variable, since stress reaction norms can be extremely plastic, allowing individuals to change their overall behavioural response while still maintaining their general interindividual ranking. Finally, we calculated a correlation matrix for the personality estimates in order to identify possible behavioural syndromes.

To test if experimental treatment affected hormone plasma concentrations, we used linear mixed models (LMM). Hormone (CORT or T) concentrations were used as dependent variables, with individuals used as a random factor and group (CT, IT), sample (baseline, 12h, 24d, 47d), the opposing hormone, and eating behaviour (individuals which presented normal behaviour vs. individuals who had to be force fed) as fixed factors. Model diagnostics to assess dispersion and distribution of scaled residuals were performed using the 'DHARMa' package (Hartig, 2020).

To test for the relationship between hormonal and personality estimates, we calculated personality estimates as the mean values for each individual in the first three behavioural trials. We did not include behavioural measures taken after temperature increase to avoid conflicting results due to possible behavioural changes that could arise from changes in temperature. Then, we used linear models (LM) for each hormonal measure as dependent variables and all personality estimates (AD, PC, WM, TC), and the group (CT, IT) as fixed factors. We included in the model analysis every possible permutation between the four personality estimates and the different groups, including interaction terms between personality and group.

To test how hormones and personality might have affected changes in body condition throughout the experiment, we used LMs including change in BI as a dependent variable and eating behaviour, group, personality estimates and hormone levels after 24d of stress as fixed factors. We chose the 24d measure because it was when groups presented a larger difference in hormone levels (Table 2.1).

All model analyses were performed with the 'glmmTMB' package and models were selected if ΔAICc ≤ 2.0. We also calculated the 95% confidence interval for each parameter in the selected models. A parameter was considered significant if the confidence interval did not cross over zero. All analyses were made using R software, version 4.2.1.

Table 2.1: Mean values and standard deviations for all hormone and behavioural measures. Hormone data is divided by samples, behavioural data is divided by trials, both data are divided by experimental group.

Hormone	Sample	CT	IT
CORT	Baseline	1.83 (± 1.38)	2.94 (± 2.12)
	12h	0.61 (± 0.64)	1.97 (± 1.40)
	24d	0.15 (± 0.21)	0.99 (± 1.21)
	47d	0.17 (± 0.13)	0.52 (± 0.47)
T	Baseline	7.54 (± 3.28)	5.57 (± 2.85)
	12h	4.35 (± 4.54)	1.37 (± 0.75)
	24d	3.86 (± 2.99)	0.52 (± 0.34)
	47d	1.06 (± 0.56)	0.34 (± 0.17)
Behaviour	Trials	CT	IT
AD	All [1-6]	8.93 (± 8.70)	5.13 (± 6.03)
	Before [1-3]	8.79 (± 9.27)	6.15 (± 6.52)
	After [4-6]	9.07 (± 8.20)	4.10 (± 5.38)
PC	All [1-6]	36.47 (± 30.47)	21.81 (± 16.88)
	Before [1-3]	32.55 (± 32.56)	23.50 (± 16.76)
	After [4-6]	40.39 (± 28.06)	20.11 (± 17.02)
WM	All [1-6]	27.13 (± 31.15)	17.95 (± 20.47)
	Before [1-3]	28.50 (± 34.37)	22.00 (± 22.67)
	After [4-6]	25.75 (± 27.91)	13.91 (± 17.33)
TC	All [1-6]	448.18 (± 399.31)	220.28 (± 335.14)
	Before [1-3]	434.30 (± 409.88)	225.05 (± 292.34)
	After [4-6]	462.07 (± 392.70)	215.52 (± 376.47)

CT = constant temperature group, IT = increment temperature group, CORT = corticosterone, T = testosterone, Baseline = baseline samples collected 15 days after habituation, 12h = sample collected 12 hours after temperature increase, 24d = sample collected 24 days after temperature increase, 47d = samples collected 47 days after temperature increase, All = values for all six behavioural trials, Before = values for behavioural trials before temperature increase, After = values for behavioural trials after temperature increase, AD = ambulatory distance, PC = posture change, WM = wall movements, TC = time on centre of the arena.

2.4. RESULTS

2.4.1. Corticosterone and testosterone plasma levels

The selected models which explained our hormonal data (ΔAICc $\leq$ 2.0) can be found on Table 2.2, values of estimates and confidence intervals for each parameter in the models can be found on Table 2.3 Experimental treatment increased levels of CORT (Fig. 2.2a) and decreased levels of T (Fig. 2.2b) in all samples collected throughout the experiment in the IT group. The magnitude of effect was larger on samples collected 24 days after temperature increase, with levels of CORT 6.6 times higher and levels of T 7.4 times lower in the IT group on average. Furthermore, there was an overall decrease on plasma levels of both hormones throughout the experiment (Fig. 2.2, Table 2.1).

Eating behaviour affected levels of T but not CORT (Table 2.2, Fig. 2.3). Animals that displayed the expected eating behaviour also had higher levels of T in comparison with animals which had to be force fed. Finally, there was an interaction between CORT and T (Table 2.2, Fig. 2.4). Animals with higher plasma CORT also had lower plasma T. However, this relationship seemed to be sample and group dependent.

Table 2.2: Fitted models from (1) linear mixed models explaining variation in hormonal data throughout the experiment, (2) linear models explaining variation in each hormone sample as a function of personality, and (3) linear models explaining variation in loss of body index throughout the experiment. Only selected models (ΔAICc ≤ 2) are shown. Results in red indicate models which only included variation due to experimental group.

Variable	Model	K	AICc	ΔAICc	Weight
1.	*Models for change in hormonal variables throughout the experiment*				
CORT	~Group + Sample + Testosterone + (1|ID)	8	350.05	0	0.88
T	~Group * Sample + EB + Corticosterone + (1|ID)	12	238.66	0	0.37
	~Group * Sample + EB + (1|ID)	11	238.7	0.04	0.36
	~Group * Sample + Corticosterone + (1|ID)	11	240.4	1.74	0.16
2.	*Models for interaction between personality and hormonal variables*				
Baseline CORT	~Group	3	79.52	0	0.31
	~PC + Group	4	80.94	1.42	0.15
	~AD + Group	4	81.5	1.97	0.12
12h CORT	~TC + WM + Group	5	91.03	0	0.16
	~TC + Group	4	92.37	1.34	0.08
	~WM + Group	4	92.57	1.54	0.08
	~AD + WM + Group	5	92.67	1.64	0.07
24d CORT	~TC + AD + WM * Group	7	98.11	0	0.14
	~TC + (AD + WM) * Group	8	99.26	1.15	0.08
	~WM * Group	5	99.51	1.4	0.07
	~TC + PC + WM * Group	7	99.75	1.64	0.06
	~TC + AD + Group	5	99.98	1.87	0.06
47d CORT	~Group	3	92.7	0	0.3
Baseline T	~TC	3	52.88	0	0.32
12h T	~TC + Group	4	70.36	0	0.33
24d T	~TC + Group	4	70.82	0	0.25
	~TC + PC + Group	5	72.58	1.75	0.1
	~TC + AD + Group	5	72.61	1.79	0.1
47d T	~TC + WM + Group	5	54.84	0	0.17
	~TC + PC + Group	5	56	1.16	0.09
	~TC + WM * Group	6	56.34	1.5	0.08
3.	*Models for change in body index throughout the experiment*				
BI change	~EB + TC + Group + 24d T	6	260.6	0	0.33
	~EB + Group + 24d CORT + 24d T	6	261.39	0.79	0.22
	~EB + TC + Group + 24d CORT + 24d T	7	261.66	1.06	0.19
	~EB + Group + 24d T	5	261.96	1.35	0.17

ID = individual, EB = eating behaviour, CORT = corticosterone, T = testosterone, AD = ambulatory distance, PC = posture change, WM = wall movements, TC = time on centre of the arena, BI = body index, Group = constant temperature vs. increment temperature groups, Sample = hormones measures taken at either baseline, 12 hours, 24 days, or 47 days after temperature increase

Table 2.3: Estimates and confidence intervals for each parameter on selected models explaining change in hormone values throughout the experiment as a function of experimental group, plasma sample and corticosterone or testosterone. Individual identity was used as a random factor. Parameters were considered significant (marked in bold) if confidence intervals did not cross over zero.

Selected models for change in hormonal variables throughout the experiment		Parameters	Estimate	Confidence Interval
CORT	~Group + Sample + T + (1|ID)	**Intercept**	0.885	**0.153 | 1.618**
		Group	0.952	**0.383 | 1.521**
		12h	-1.490	**-2.024 | -0.956**
		24d	-2.957	**-3.575 | -2.338**
		47d	-3.133	**-3.893 | -2.372**
		T	-0.362	**-0.615 | -0.109**
		ID	0.540	**0.350 | 0.834**
T	~Group * Sample + EB + CORT + (1|ID)	**Intercept**	1.721	**1.340 | 2.103**
		EB	0.421	**0.033 | 0.809**
		Group	-0.890	**-1.280 | -0.500**
		12h	-1.074	**-1.553 | -0.595**
		24d	-2.227	**-2.678 | -1.777**
		47d	-0.159	-0.649 | 0.331
		CORT	-0.087	-0.194 | 0.020
		12h:Group	-0.622	**-1.133 | -0.111**
		24d:Group	-1.562	**-2.084 | -1.040**
		47d:Group	-0.706	**-1.222 | -0.190**
		ID	0.437	**0.311 | 0.614**
	~Group * Sample + EB + (1|ID)	**Intercept**	1.679	**1.297 | 2.062**
		EB	0.455	**0.064 | 0.846**
		Group	-0.775	**-1.142 | -0.408**
		12h	-0.820	**-1.187 | -0.453**
		24d	-2.010	**-2.378 | -1.643**
		47d	-0.227	-0.715 | 0.261
		12h:Group	-0.661	**-1.175 | -0.148**
		24d:Group	-1.658	**-2.172 | -1.145**
		47d:Group	-0.744	**-1.263 | -0.225**
		ID	0.443	**0.316 | 0.622**
	~Group * Sample + CORT + (1|ID)	**Intercept**	1.895	**1.536 | 2.254**
		Group	-0.209	-0.716 | 0.299
		12h	-0.905	**-1.296 | -0.515**
		24d	-1.106	**-1.587 | -0.625**
		47d	-2.255	**-2.707 | -1.803**
		CORT	-0.097	-0.205 | 0.010
		12h:Group	-0.615	**-1.126 | -0.104**
		24d:Group	-1.549	**-2.071 | -1.026**
		47d:Group	-0.696	**-1.212 | -0.179**
		ID	0.478	**0.345 | 0.663**

ID = individual, EB = eating behaviour, CORT = corticosterone, T = testosterone, 12h = sample collected 12 hours after temperature increase, 24d = sample collected 24 days after temperature increase, 47d = samples collected 47 days after temperature increase

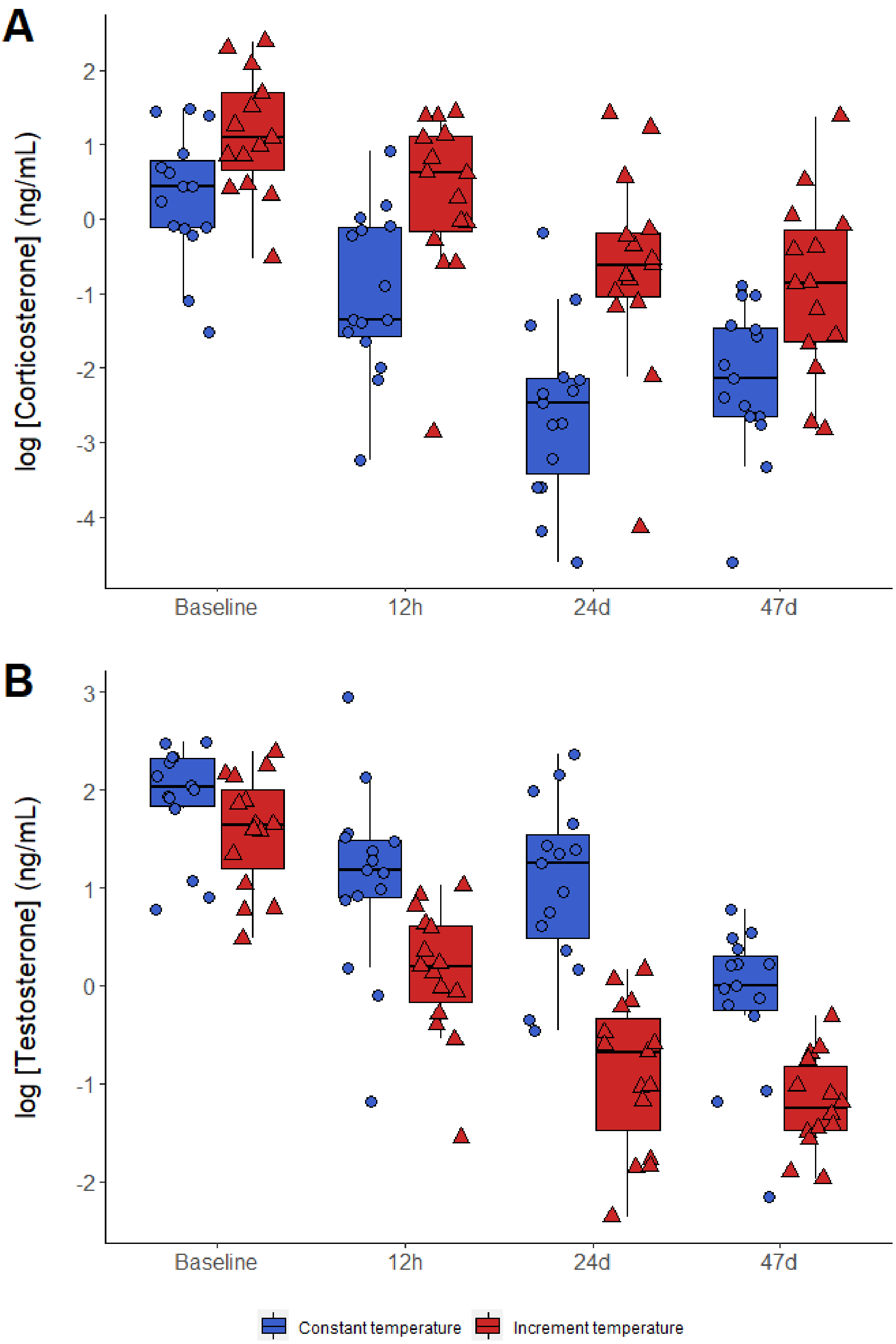

Figure 2.2: Boxplots showing values of (A) corticosterone and (B) testosterone measured in each sample. Individuals kept at constant temperature throughout the experiment are shown in blue and individuals who were exposed to high temperature stress in red. 12h, 24d and 47d respectively denote samples collected 12 hours, 24 days and 47 days after temperature increase.

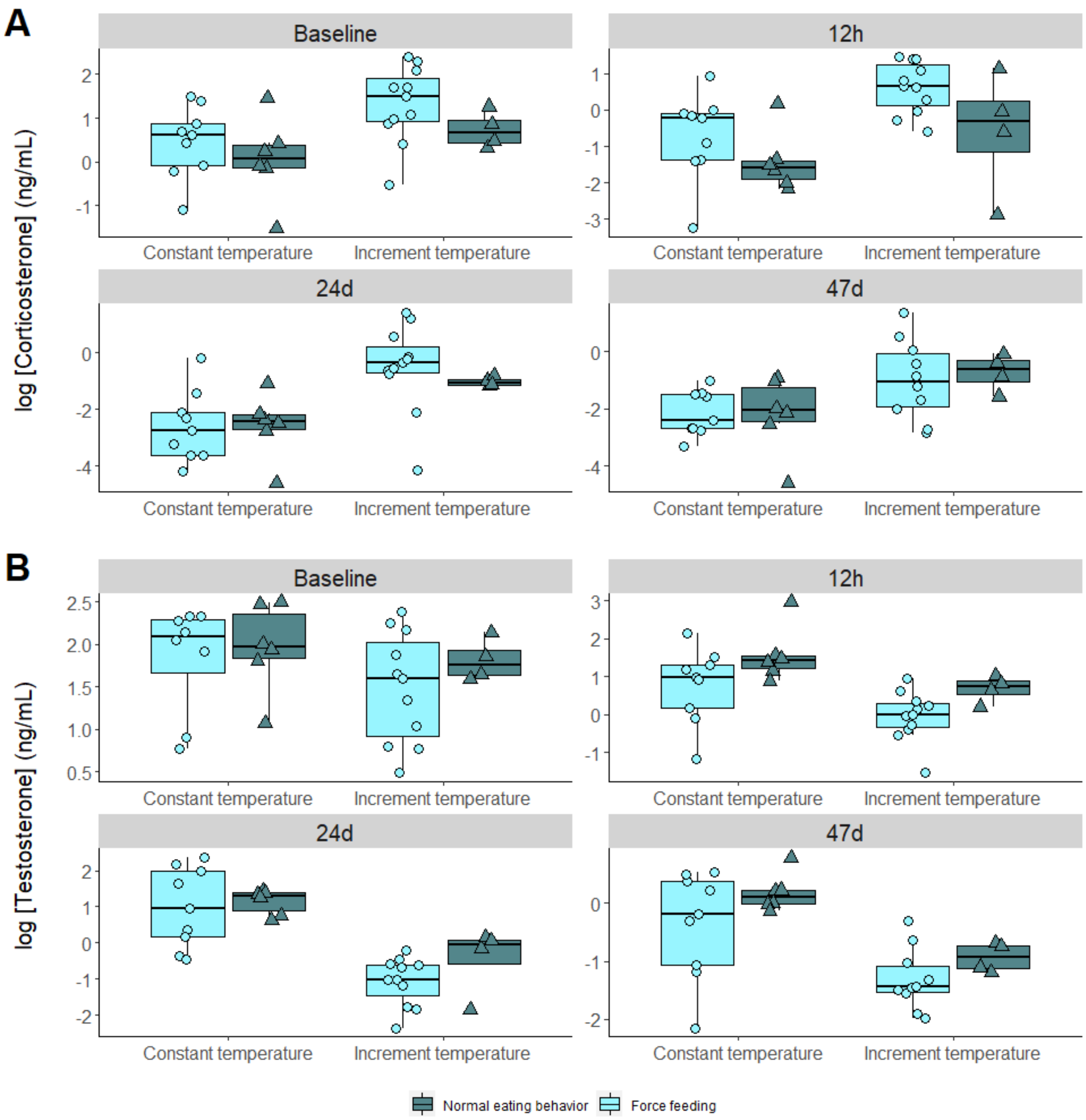

Figure 2.3: Boxplots showing relationship between eating behaviour and (A) corticosterone and (B) testosterone for each sample and experimental group. 12h, 24d and 47d respectively denote samples collected 12 hour, 24 days and 47 days after temperature increase.

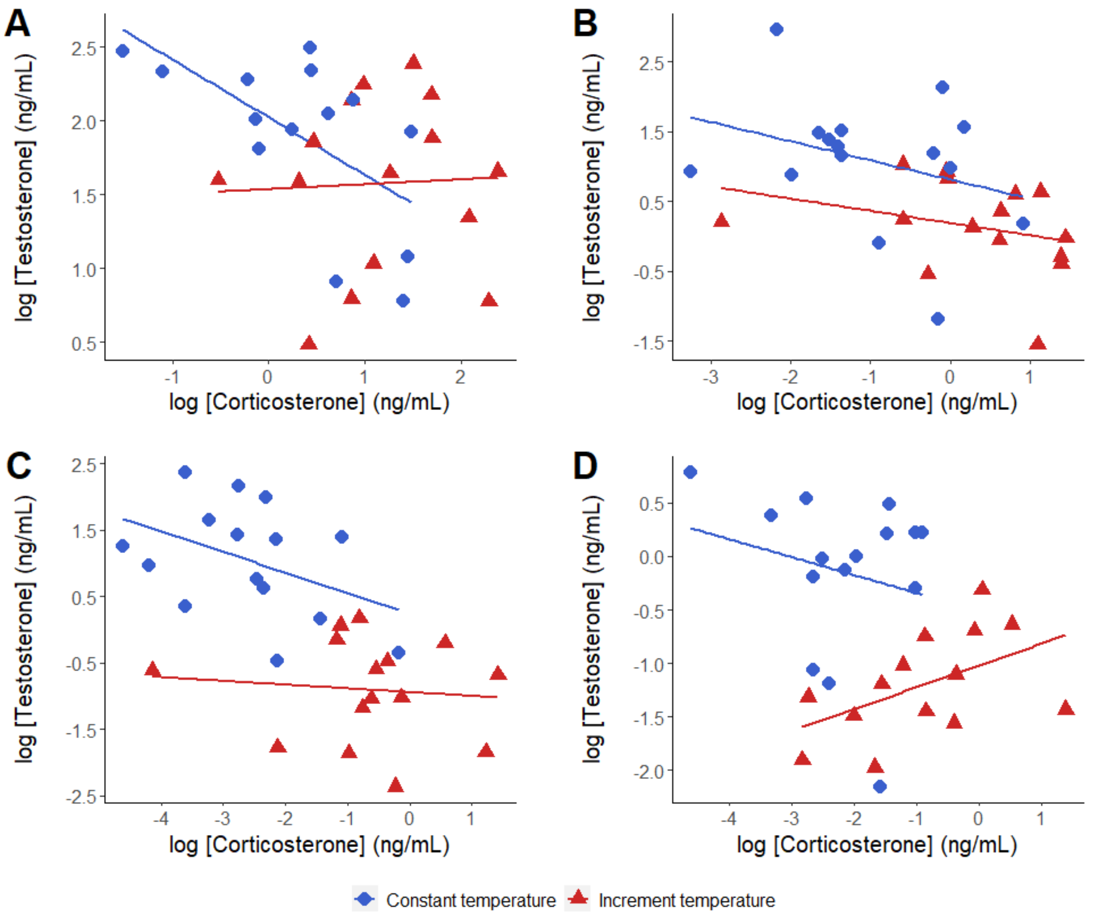

Figure 2.4: Correlations between corticosterone and testosterone for each group on (A) baseline samples, (B) samples collected 12 hours after temperature increase, (C) samples collected 24 days after temperature increase, and (D) samples collected 47 days after temperature increase.

2.4.2. Behavioural variables and animal personality

PCA identified that PC1 containing variation in AD, PC, and WM explained 63.9% of observed variance in our data, while PC2 containing only TC explained 29.1% of observed variance (Fig. 2.5). Permutations identified statistical significance of both ψ (ψ = 3.928, max null ψ = 1.506, min null ψ = 0.037, p < 0.001) and φ (φ = 0.572, max null φ = 0.354, min null φ = 0.055, p < 0.001), but only PC1 was found to be statistically significant (PC1 eigenvalue = 2.554, max null eigenvalue = 2.059, p < 0.001; PC2 eigenvalue = 1.165, max null eigenvalue = 1.399, p = 0.223). This supports that AD, PC, and WM represent variation in the same behavioural axis (exploration).

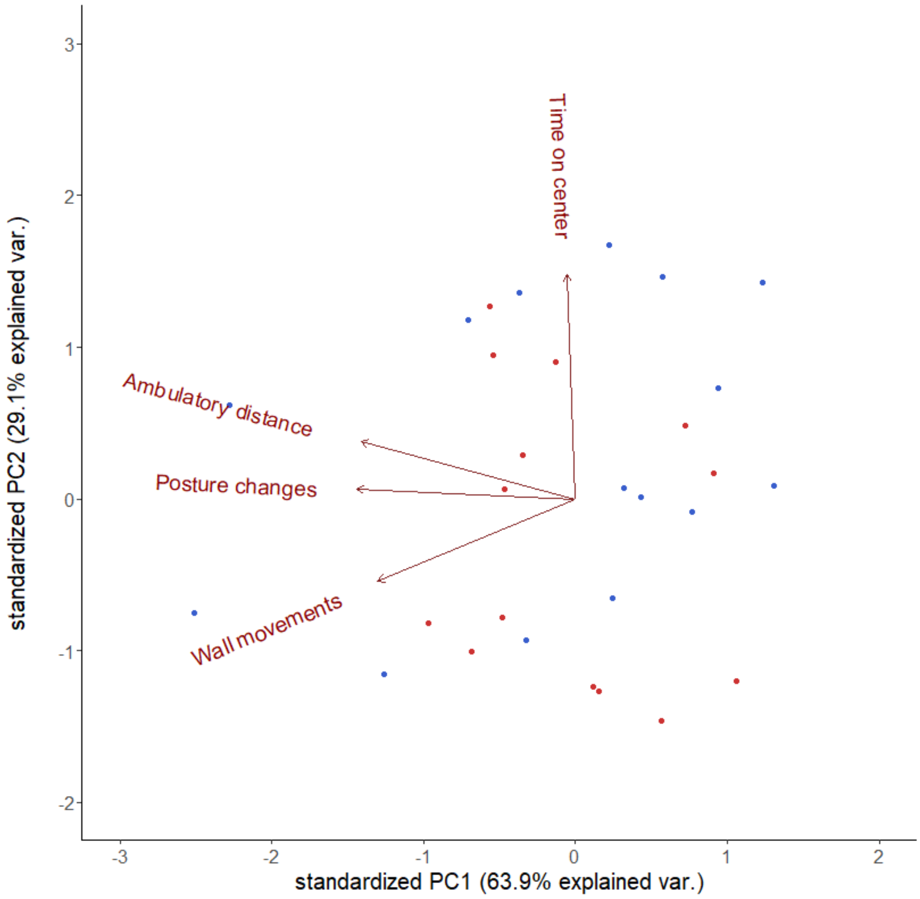

Figure 2.5: Results from a Principal Component Analysis on the four behavioural variables analysis. PC1 containing ambulatory distance, posture change and wall movements explain 63.9% of the variance in our data, while PC2 containing time on centre of the arena explains 29.1%. PC1 and PC2 combined explain 93% of the variance on behavioural variables.

Adjusted repeatability values, confidence intervals and p-values for all variables can be found on Table 2.4. Repeatability was significant ($p < 0.05$) for all variables both when considering all six trials and when separating the analysis between before (trials 1-3) and after (trials 4-6) temperature treatment. Notably, repeatability estimates were lower for all variables when all six trials were grouped into the analysis (Table 2.4).

Pearson's correlation coefficient, t-values, and p-values for change in personality estimates and Spearman's rank order correlation coefficient, S values and p-values for change in rank order before and after experimental treatment can be found on Table 2.5. There was no significant correlation between AD before and after treatment in either group. PC and WM showed significant correlations before and after for the CT group, but not IT. TC showed significant correlations before and after for both groups (Table 2.5, Fig. 2.6-2.7 a,c,e,g). When comparing rank orders, results are the same, except that AD rank order before and after was also significantly correlated in the control group and WM rank order before and after was significantly correlated in both groups (Table 2.5, Fig. 2.6-2.7 b,d,f,h).

Table 2.4: Adjusted repeatability estimates, confidence intervals and p-values for each behavioural variable measured. Estimates are shown for all trials combined, as well as separately for trials before and after temperature was increased. Significance is marked in bold.

Behaviour	Trials	Repeatability	CI	p-value
AD	All [1-6]	**0.333**	[0.157, 0.487]	**1.48E-07**
	Before [1-3]	**0.368**	[0.125, 0.592]	**1.60E-03**
	After [4-6]	**0.487**	[0.232, 0.682]	**2.73E-05**
PC	All [1-6]	**0.336**	[0.154, 0.503]	**1.01E-07**
	Before [1-3]	**0.401**	[0.174, 0.632]	**5.71E-04**
	After [4-6]	**0.442**	[0.192, 0.655]	**1.79E-04**
WM	All [1-6]	**0.369**	[0.196, 0.537]	**1.21E-08**
	Before [1-3]	**0.406**	[0.153, 0.633]	**6.33E-04**
	After [4-6]	**0.381**	[0.134, 0.601]	**1.60E-03**
TC	All [1-6]	**0.550**	[0.374, 0.685]	**9.65E-17**
	Before [1-3]	**0.562**	[0.313, 0.742]	**7.41E-07**
	After [4-6]	**0.649**	[0.450, 0.789]	**3.84E-08**

AD = Ambulatory distance, PC = Posture change, WM = Wall movements, TC = Time on centre of the arena, CI = confidence interval.

Table 2.5: Pearson's correlation coefficient (r), t-values, and p-values for each personality estimates and Spearman's rank order correlation coefficient (ρ), S values and p-values for each individual's rank order before and after temperature increase. Significance is marked in bold. Sample size for every correlation was 15 individuals (N = 15).

Variable	Group	r	T	p-value
AD	CT	0.512	2.147	0.051
	IT	0.391	1.530	0.150
PC	CT	0.772	4.377	**7.494E-04**
	IT	0.210	0.773	0.453
WM	CT	0.806	4.912	**2.838E-04**
	IT	0.464	1.889	0.081
TC	CT	0.577	2.545	**0.024**
	IT	0.849	5.800	**6.175E-05**
Variable	Group	ρ	S	p-value
AD rank	CT	0.525	266	**0.047**
	IT	0.352	363	0.198
PC rank	CT	0.550	252	**0.036**
	IT	0.054	530	0.853
WM rank	CT	0.757	136	**0.002**
	IT	0.546	254	**0.038**
TC rank	CT	0.539	258	**0.041**
	IT	0.828	97	**1.403E-04**

AD = Ambulatory distance, PC = Posture change, WM = Wall movements, TC = Time on centre of the arena, Rank = rank order for corresponding personality estimate, CT = Constant temperature group, IT = Increment temperature group.

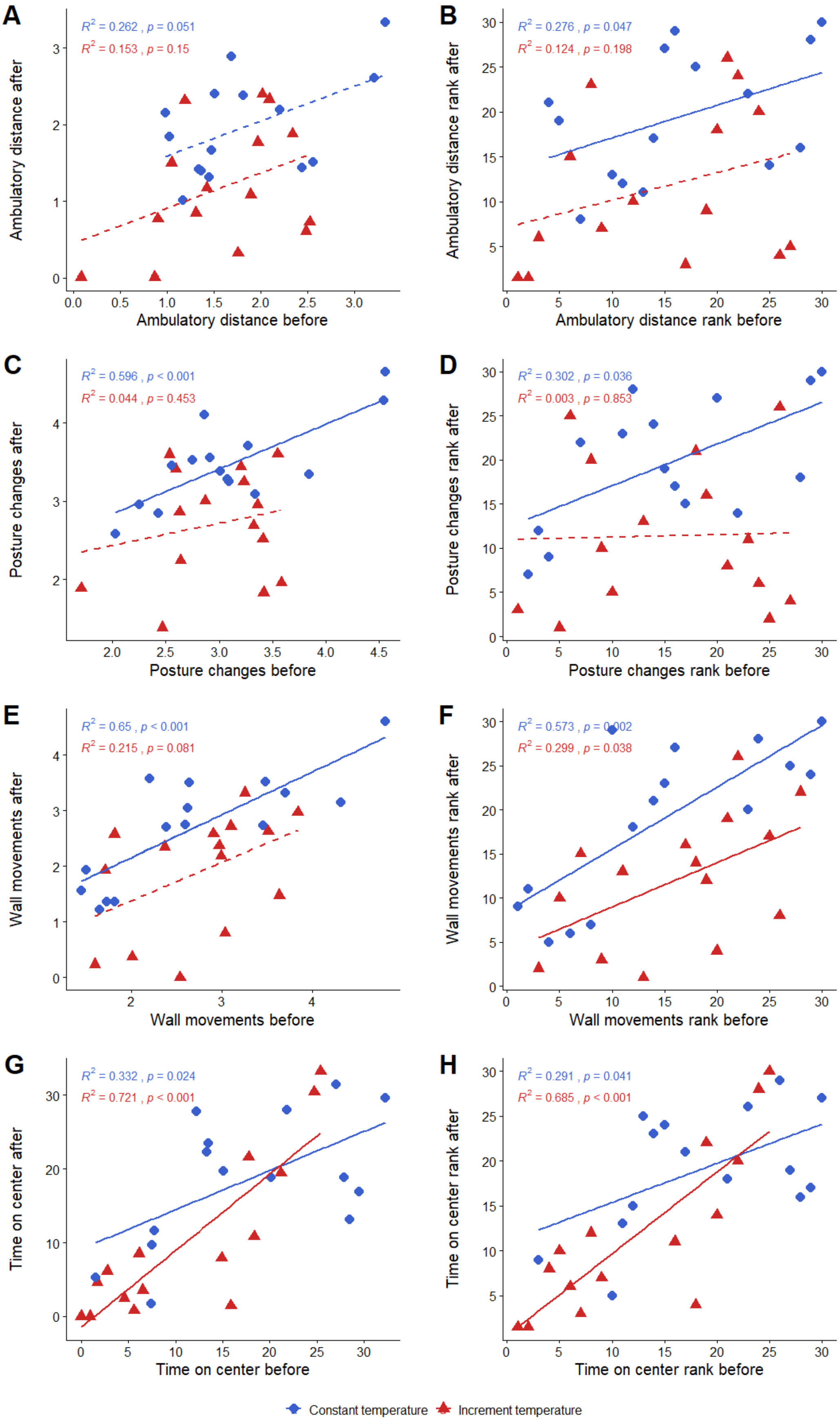

Figure 2.6: Correlations between (left) personality estimates and (right) each individual's rank order before and after temperature increase. R^2 and p-values are shown. Dashed lines indicate non-significant correlations ($p > 0.05$).

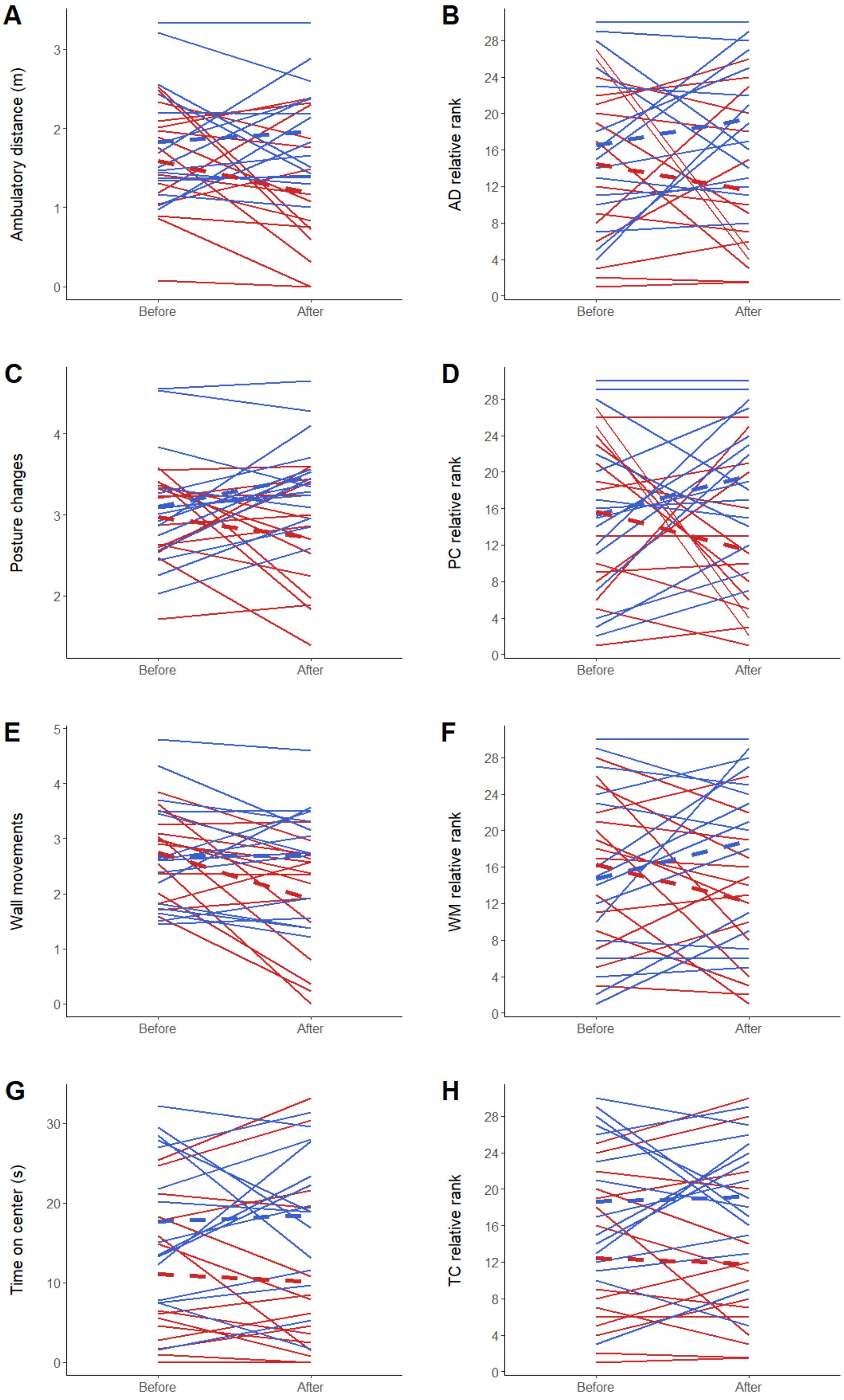

Figure 2.7: Individual reaction norms for (left) personality variables and (right) each individual's rank order before and after temperature increase. Blue lines denote individuals from the constant temperature group and red lines denote individuals from the increment temperature group. Dashed lines denote group averages.

Personality estimates for exploratory variables (AD, PC, WM) were positively correlated between each other, but not with TC (ADxPC: $r = 0.892$, $t = 10.423$, $p < 0.001$; ADxWM: $r = 0.684$, $t = 4.965$, $p < 0.001$; ADxTC: $r = 0.254$, $t = 1.389$; $p = 0.176$, PCxWM: $r = 0.749$, $t = 5.989$, $p < 0.001$; PCxTC: $r = 0.054$, $t = 0.285$, $p = 0.778$; WMxTC: $r = -0.268$, $t = -1.473$, $p = 0.152$, Fig. 2.8).

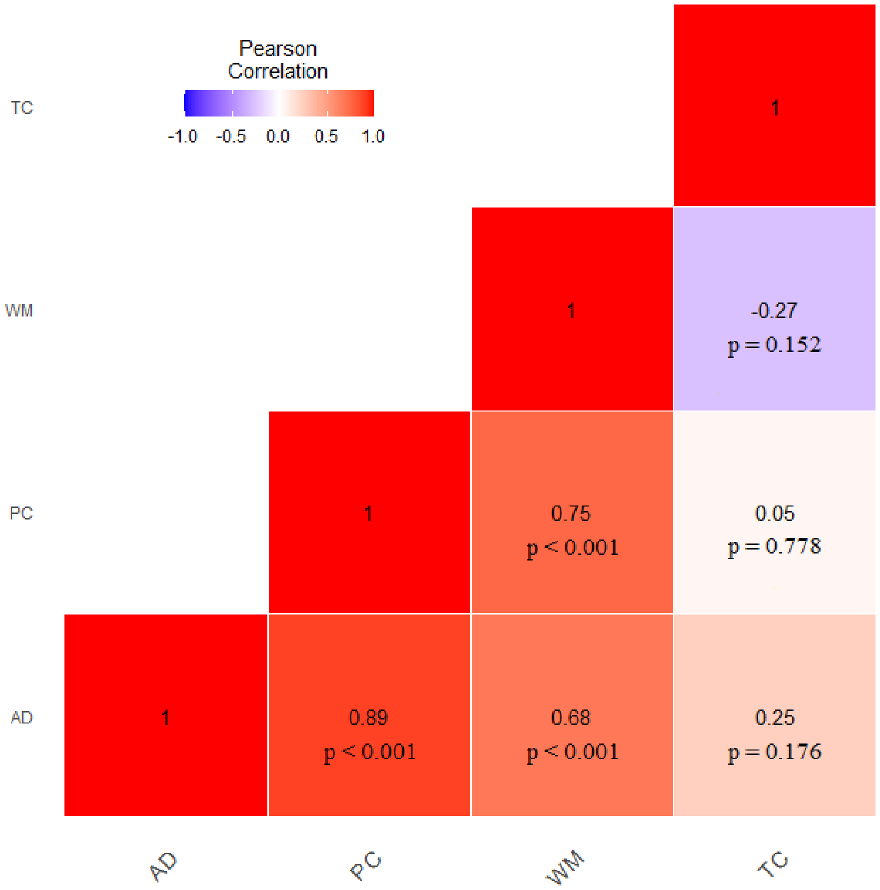

Figure 2.8: Correlation matrix between each personality variable. AD = Ambulatory distance, PC = Posture change, WM = Wall movements, TC = Time on centre of the arena.

2.4.3. Personality x Hormonal correlates

Selected models which best explained the relationships between hormones and personality (ΔAICc ≤ 2.0) can be found on Table 2.2, as well as the values of estimates and confidence intervals for each parameter on Table 2.6. Correlation between the different variables was highly dependent on the hormone sample, personality estimate, and the experimental group (Fig. 2.9-2.12).

Table 2.6: Estimates and confidence intervals for each parameter on selected models explaining variation in hormonal variables as a function of personality variables. Parameters were considered significant (marked in bold) if confidence intervals did not cross over zero. Results in red indicate models which only included variation due to experimental group.

Selected models for interaction between personality and hormonal variables		Parameters	Estimate	Confidence Interval
Baseline CORT	~Group	Intercept	0.299	-0.111 | 0.710
		Group	0.860	**0.280 | 1.441**
	~PC + Group	Intercept	-0.524	-2.000 | 0.953
		PC	0.265	-0.193 | 0.724
		Group	0.895	**0.324 | 1.467**
	~AD + Group	Intercept	-0.025	-0.881 | 0.831
		AD	0.177	-0.234 | 0.588
		Group	0.903	**0.321 | 1.486**
12h CORT	~TC + WM + Group	Intercept	-1.390	-2.827 | 0.048
		TC	-0.041	**-0.078 | -0.004**
		WM	0.418	**0.034 | 0.802**
		Group	0.990	**0.306 | 1.674**
	~TC + Group	Intercept	-0.072	-0.903 | 0.760
		TC	-0.052	**-0.090 | -0.014**
		Group	0.943	**0.211 | 1.676**
	~WM + Group	**Intercept**	-2.433	**-3.609 | -1.257**
		WM	0.535	**0.137 | 0.933**
		Group	1.255	**0.564 | 1.945**
	~AD + WM + Group	**Intercept**	-2.250	**-3.392 | -1.109**
		AD	-0.579	-1.240 | 0.083
		WM	0.862	**0.329 | 1.395**
		Group	1.093	**0.409 | 1.778**
24d CORT	~TC + AD + WM * Group	Intercept	-1.567	-3.305 | 0.170
		TC	-0.068	**-0.115 | -0.021**
		AD	1.065	**0.241 | 1.890**
		Group	-1.828	-4.185 | 0.530
		WM	-0.660	-1.427 | 0.106
		WM:Group	1.316	**0.491 | 2.141**
	~TC + (AD + WM) * Group	Intercept	-1.562	-3.225 | 0.102
		AD	2.070	**0.640 | 3.500**
		WM	-1.310	**-2.375 | -0.246**
		Group	-1.635	-3.903 | 0.634
		TC	-0.074	**-0.119 | -0.028**
		AD:Group	-1.249	-2.732 | 0.233
		WM:Group	2.058	**0.875 | 3.241**
	~WM * Group	**Intercept**	-3.195	**-4.675 | -1.714**
		WM	0.225	**-0.290 | 0.740**
		Group	-0.903	-3.508 | 1.702
		WM:Group	1.028	**0.114 | 1.943**

Table 2.6 continued:

24d CORT	~TC + PC + WM * Group	**Intercept**	-3.340	**-5.235 | -1.444**
		TC	-0.049	**-0.090 | -0.007**
		PC	0.987	**0.073 | 1.901**
		Group	-1.836	-4.279 | 0.607
		WM	-0.539	-1.319 | 0.241
		WM:Group	1.316	**0.459 | 2.172**
	~TC + AD + Group	**Intercept**	-2.978	**-4.211 | -1.746**
		TC	-0.053	**-0.095 | -0.011**
		AD	0.725	**0.180 | 1.270**
		Group	1.765	**0.969 | 2.561**
47d CORT	~Group	**Intercept**	-2.164	**-2.701 | -1.627**
		Group	1.259	**0.487 | 2.032**
Baseline T	~TC	**Intercept**	1.391	**1.038 | 1.743**
		TC	0.023	**0.003 | 0.043**
12h T	~TC + Group	Intercept	0.230	-0.346 | 0.806
		TC	0.048	**0.022 | 0.075**
		Group	-0.629	**-1.137 | -0.122**
24d T	~TC + Group	Intercept	0.224	-0.357 | 0.805
		TC	0.046	**0.020 | 0.073**
		Group	-1.641	**-2.153 | -1.129**
	~TC + PC + Group	Intercept	0.870	-0.431 | 2.170
		TC	0.046	**0.020 | 0.073**
		PC	-0.210	-0.590 | 0.170
		Group	-1.667	**-2.171 | -1.163**
	~TC + AD + Group	Intercept	0.514	-0.267 | 1.295
		TC	0.049	**0.023 | 0.076**
		AD	-0.187	-0.533 | 0.158
		Group	-1.667	**-2.171 | -1.162**
47d T	~TC + WM + Group	Intercept	0.092	-0.724 | 0.908
		TC	0.027	**0.006 | 0.049**
		WM	-0.270	**-0.485 | -0.055**
		Group	-0.824	**-1.223 | -0.424**
	~TC + PC + Group	Intercept	0.264	-0.774 | 1.302
		TC	0.034	**0.013 | 0.055**
		PC	-0.328	**-0.626 | -0.030**
		Group	-0.847	**-1.256 | -0.437**
	~TC + WM * Group	Intercept	0.367	-0.523 | 1.258
		TC	0.026	**0.005 | 0.047**
		WM	-0.365	**-0.617 | -0.113**
		Group	-1.637	**-2.898 | -0.375**
		WM:Group	0.294	-0.140 | 0.727

CORT = corticosterone, T = testosterone, 12h = sample collected 12 hours after temperature increase, 24d = sample collected 24 days after temperature increase, 47d = samples collected 47 days after temperature increase, AD = ambulatory distance, PC = posture changes, WM = wall movements, TC = time on centre of the arena.

Both baseline CORT and 47d CORT had the models containing just the differences between the groups selected as the best fit, and although other models were also selected for baseline CORT, the parameters were not significant (Table 2.2 & 2.6). For the 12h measure, AD was selected in one model, but the parameter was not significant, WM and TC consistently showed up in model selection, with animals that had higher CORT levels spending less time on the centre of the arena and performing more movements against the wall (Table 2.2 & 2.6, Fig. 2.10-2.12 c). Finally, the 24d measure was correlated with all personality estimates. Animals that had higher levels of CORT travelled longer distances, had more changes in posture and movements against the wall and spent less time in the centre of the arena (Table 2.2 & 2.6, Figs. 2.9-2.12e).

Overall, T showed a consistent relationship with TC, with animals that spent more time on the centre of the arena also having higher levels of T, regardless of group or measure (Table 2.2, Fig. 2.12 b,d,f,g). Testosterone did not show a consistent relationship with the exploratory variables, but models containing AD, PC and WM were selected after animals were exposed chronically exposed to elevated temperatures on measures 24d and 47d (Table 2.2, Fig. 2.9-2.11 f,h), and this relationship was group dependent.

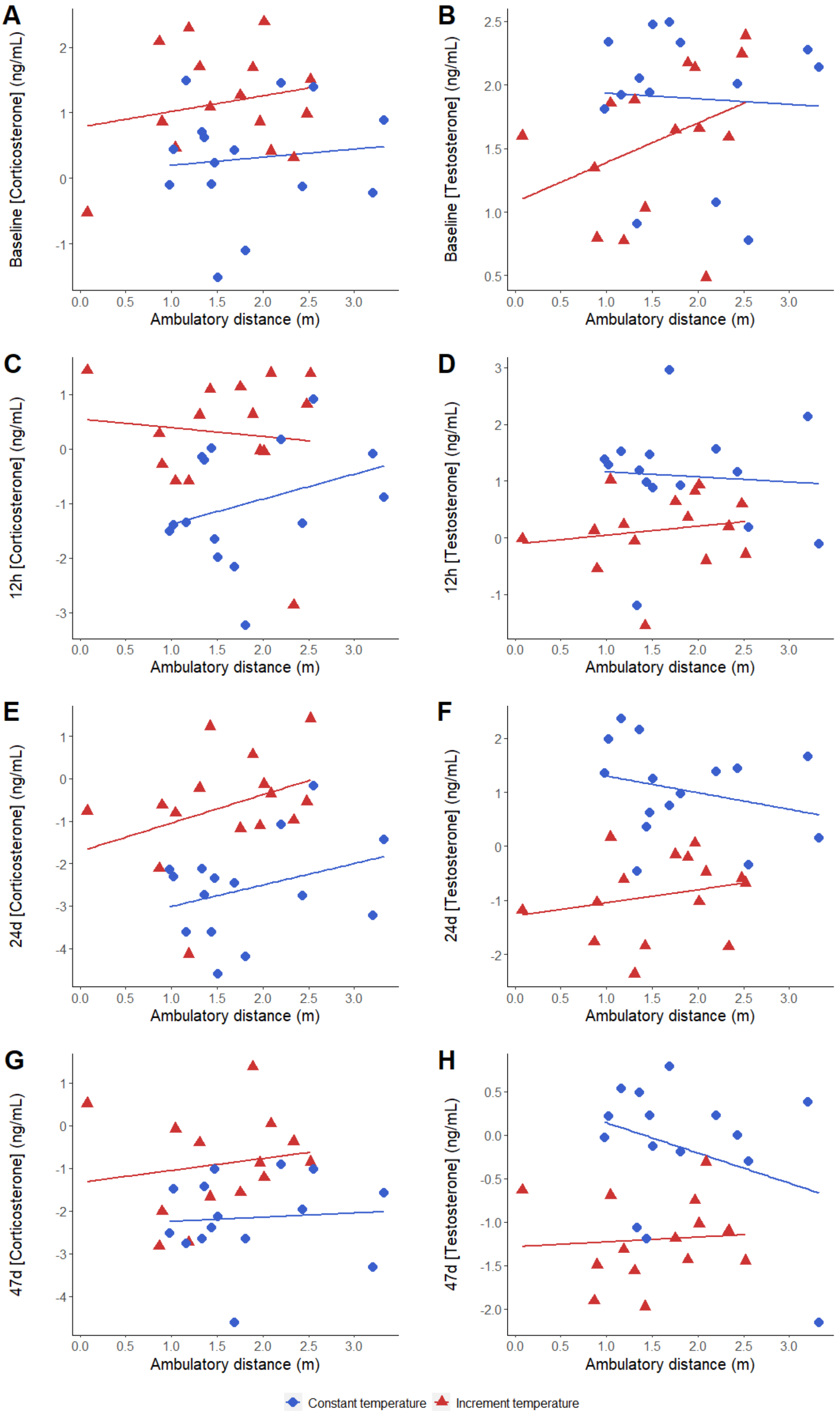

Figure 2.9: Correlations between personality estimates for ambulatory distance and each (left) corticosterone and (right) testosterone sample. 12h, 24d and 47d respectively denote samples collected 12 hour, 24 days and 47 days after temperature increase.

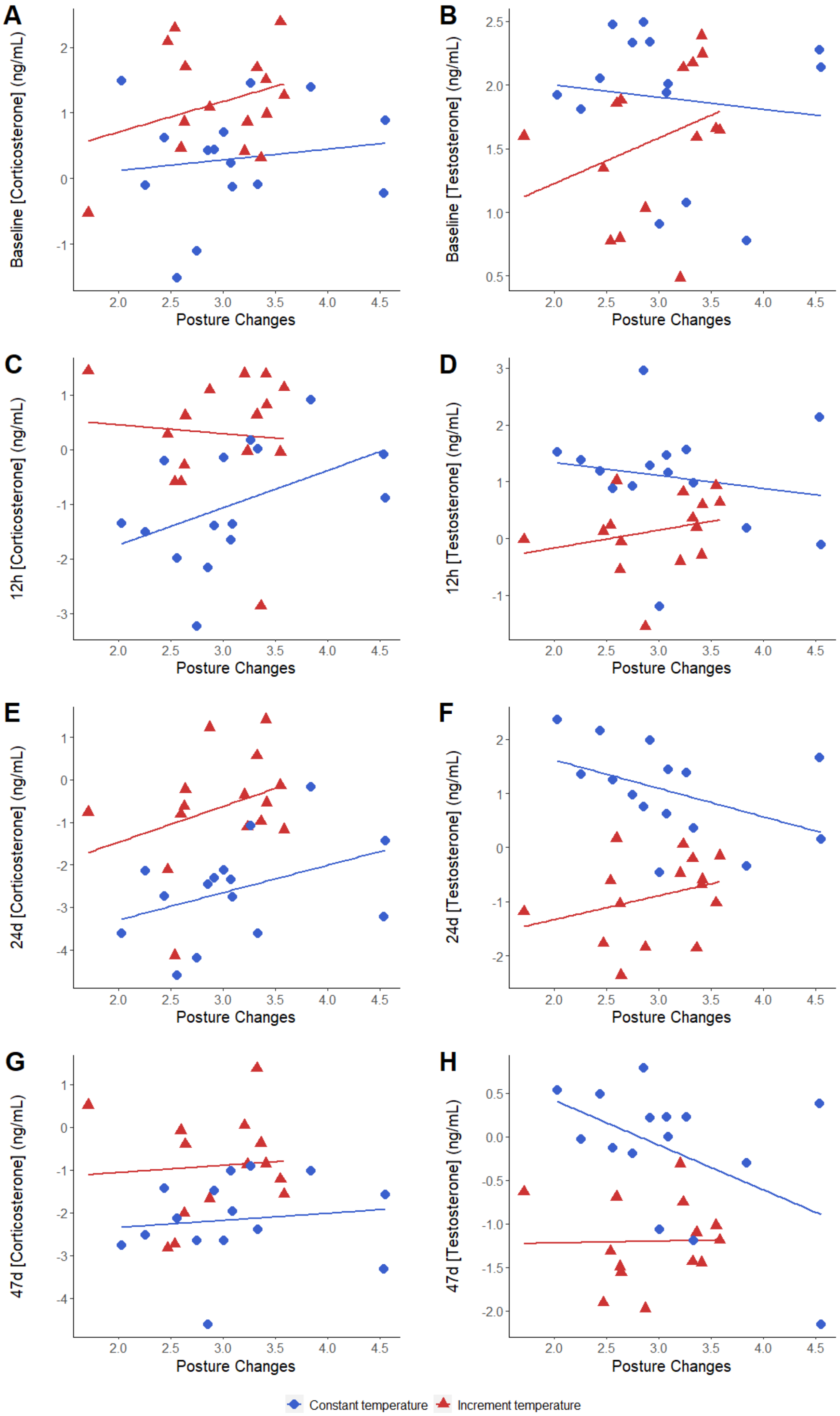

Figure 2.10: Correlations between personality estimates for changes in posture and each (left) corticosterone and (right) testosterone sample. 12h, 24d and 47d respectively denote samples collected 12 hour, 24 days and 47 days after temperature increase.

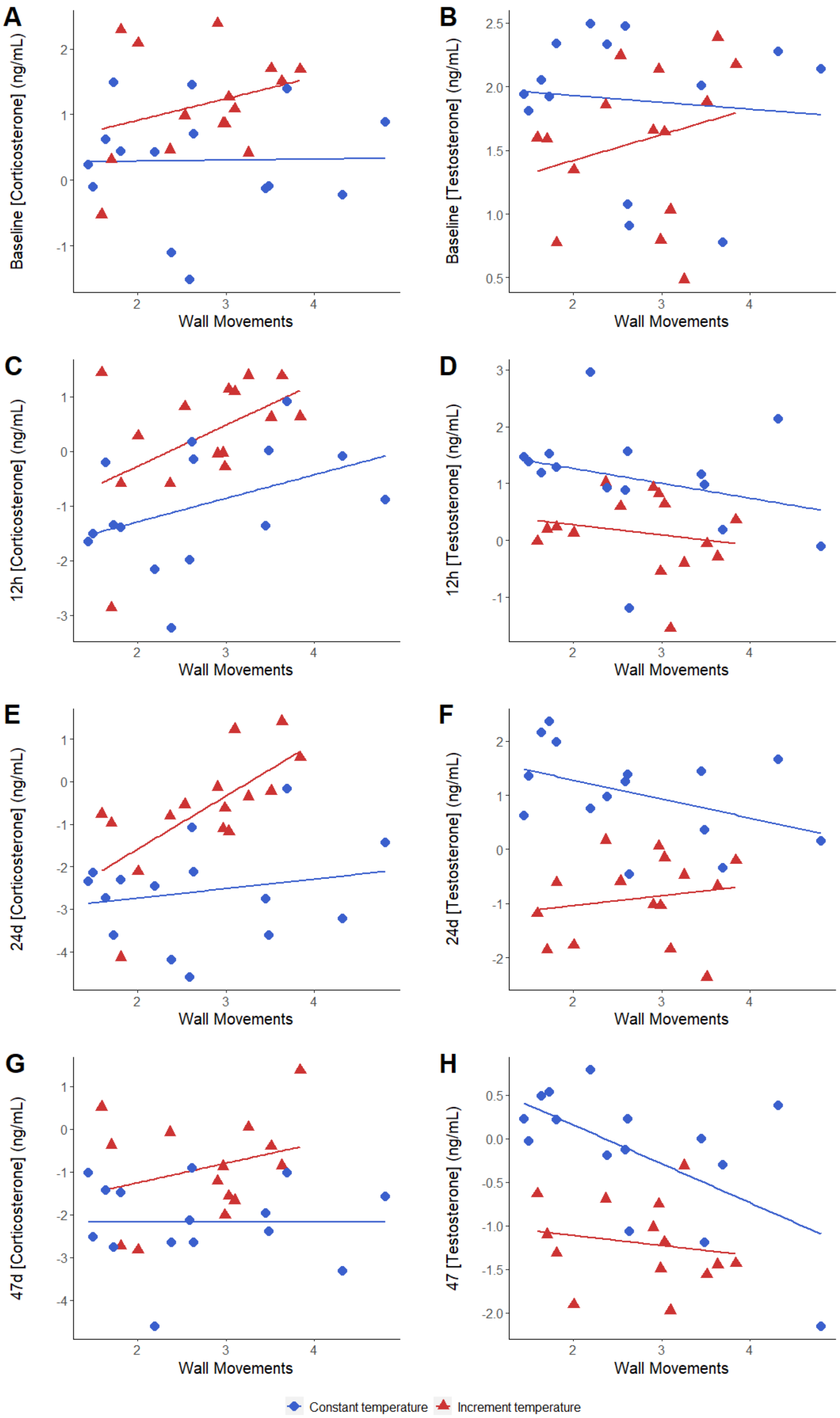

Figure 2.11: Correlations between personality estimates for movements against the wall and each (left) corticosterone and (right) testosterone sample. 12h, 24d and 47d respectively denote samples collected 12 hour, 24 days and 47 days after temperature increase.

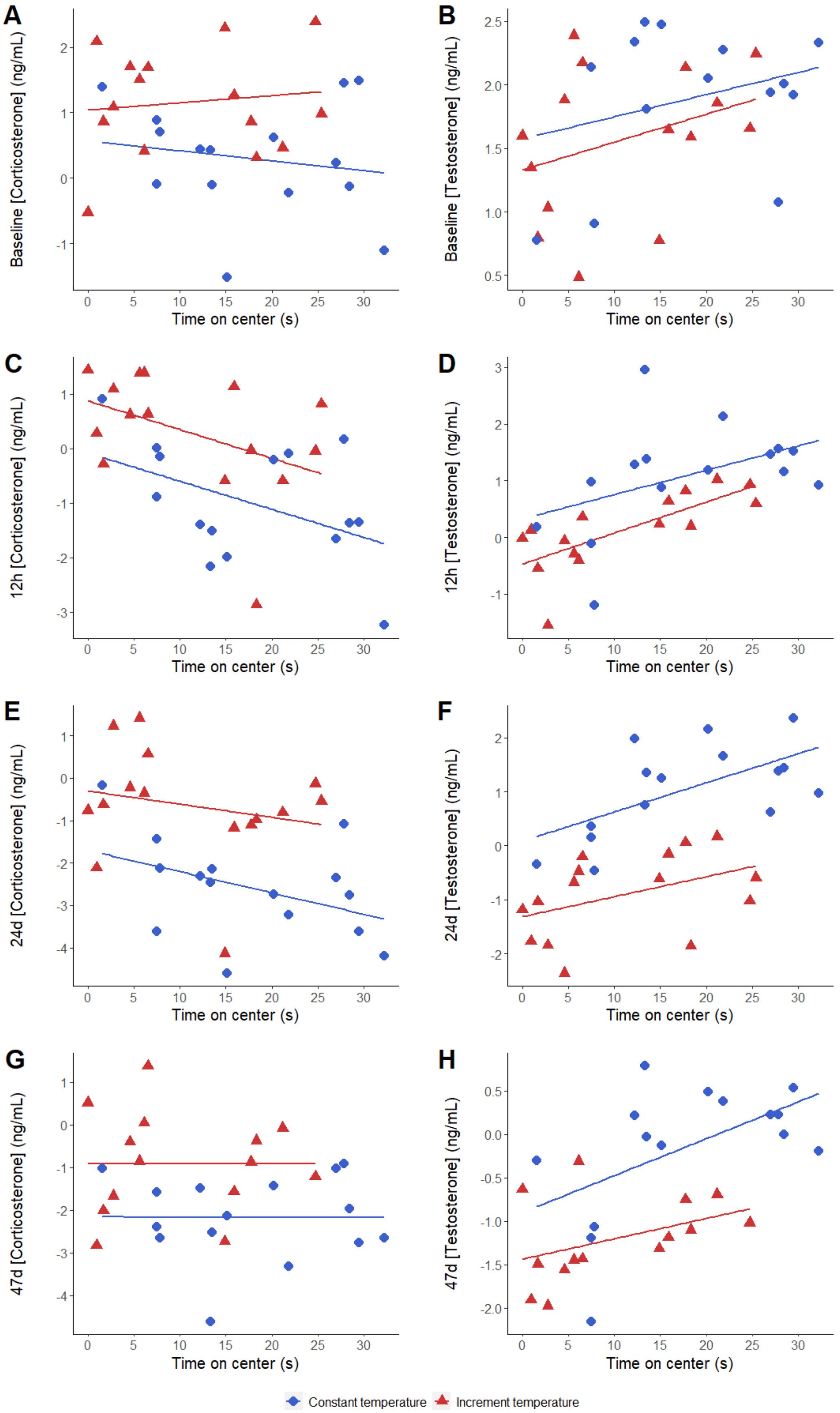

Figure 2.12: Correlations between personality estimates for time spent on centre of the arena and each (left) corticosterone and (right) testosterone sample. 12h, 24d and 47d respectively denote samples collected 12 hour, 24 days and 47 days after temperature increase.

2.4.4. Change in body index

Selected models which best explained loss in BI throughout the experiment (ΔAICc ≤ 2.0) can be found on Table 2.2, as well as the values of estimates and confidence intervals for each parameter on Table 2.7. In general, individuals that presented normal eating behaviour also had a smaller change in BI when compared with individuals that were force fed, particularly in the IT group which showed considerable loss in BI even in individuals that ate normally (Fig. 2.13). Regarding behaviour, individuals that spent less time on the centre of the arena also had a larger change in BI, but this was not true for individuals in the control group which had normal eating behaviour (Table 2.2, Fig. 2.13d). Change in BI was also correlated with hormonal variables. Individuals that had higher levels of CORT and lower levels of T on samples 24d also lost more mass. However, this relationship was only apparent in individuals that were simultaneously treated with high temperatures and force fed (Table 2.2, Fig. 2.13e,f).

Table 2.7: Estimates and confidence intervals for each parameter on selected models explaining change in body index throughout the experiment. Parameters were considered significant (marked in bold) if confidence intervals did not cross over zero.

Selected models for change in body index throughout the experiment		Parameters	Estimate	Confidence Interval
BI change	~EB + TC + Group + 24d T	**Intercept**	-34.321	**-49.111 | -19.531**
		EB	28.952	**14.858 | 43.046**
		TC	0.990	**0.117 | 1.862**
		Group	15.305	-3.948 | 34.558
		24d T	18.625	**9.799 | 27.450**
	~EB + Group + 24d CORT + 24d T	**Intercept**	-35.703	**-52.077 | -19.329**
		EB	34.727	**21.508 | 47.947**
		Group	24.276	**4.030 | 44.521**
		24d CORT	-5.229	**-10.335 | -0.123**
		24d T	21.552	**13.526 | 29.577**
	~EB + TC + Group + 24d CORT + 24d T	**Intercept**	-41.395	**-58.006 | -24.784**
		EB	29.786	**16.239 | 43.334**
		TC	0.816	-0.047 | 1.679
		Group	20.559	**1.016 | 40.101**
		24d CORT	-4.070	-9.052 | 0.911
		24d T	17.924	**9.420 | 26.427**
	~EB + Group + 24d T	**Intercept**	-24.375	**-37.258 | -11.492**
		EB	35.022	**20.918 | 49.127**
		Group	18.172	-2.476 | 38.820
		24d T	23.565	**15.261 | 31.869**

BI = body index, EB = eating behaviour, CORT = corticosterone, T = testosterone, 24d = sample collected 24 days after temperature increase, TC = time on centre of the arena.

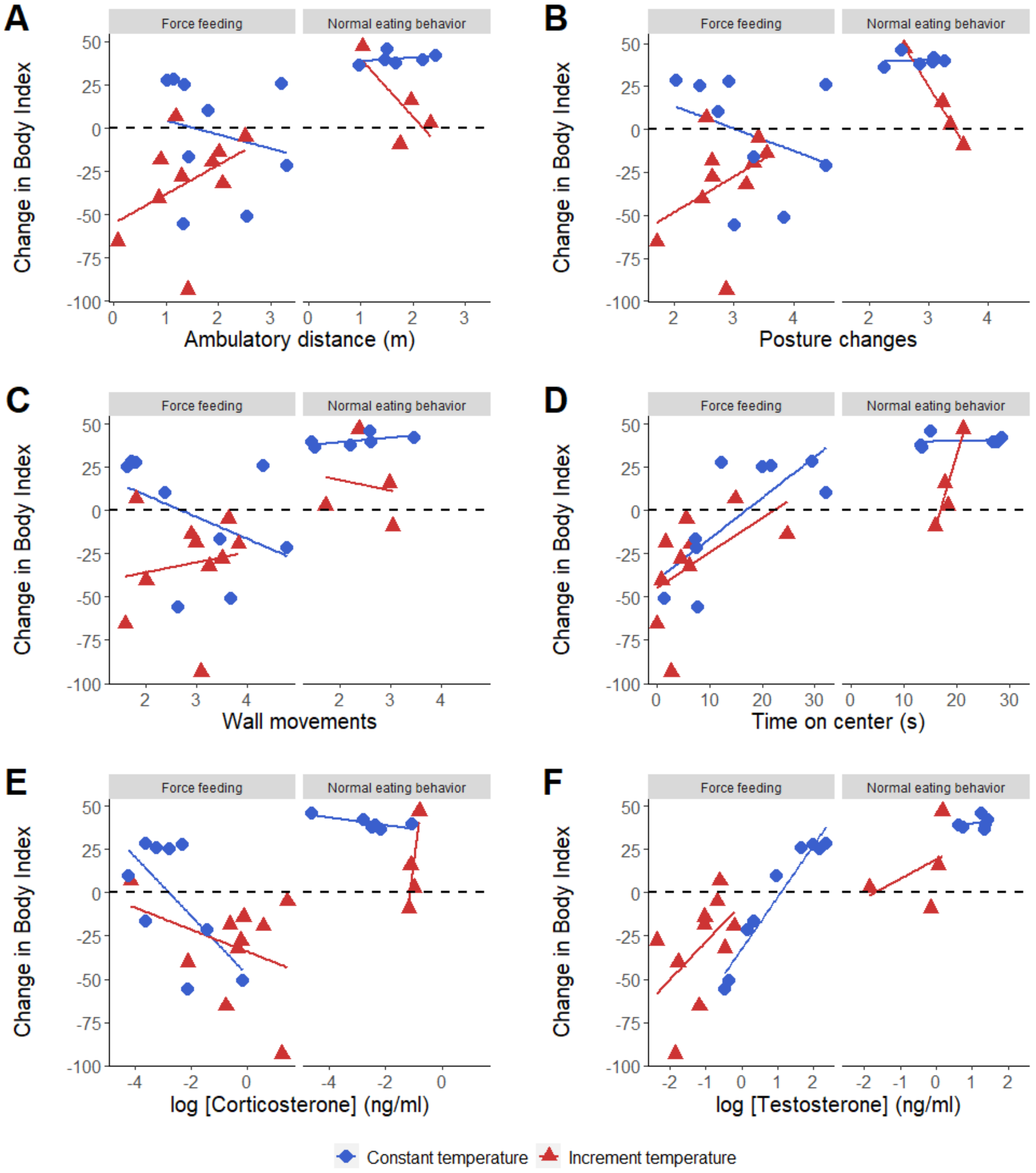

Figure 2.13: Correlations between change in body index throughout the experiment and (A) ambulatory distance, (B) posture changes, (C) wall movements, (D) time on centre of the arena, (E) plasma corticosterone concentration 24 days after temperature increase, and (F) plasma testosterone concentration 24 days after temperature increase. Data is shown for individuals that were force fed (on the left of each graph) and individuals who displayed normal eating behaviour (on the right of each graph). Horizontal dashed line indicates null change in BI, individuals above the line gained mass throughout the experiment, while individuals below the line lost mass.

2.5. DISCUSSION

Our study aimed at investigating the relationship between high-temperature stress and animal personality in the context of climate change. Amphibians are among the clades under highest vulnerability to climate change (Blaustein et al., 2010). Our hypothesis that increment temperature would constitute a potent stressor for *L. catesbeianus* for prolonged periods of time was supported. Animals exposed to high temperatures showed hormonal responses as soon as 12 hours after exposure and maintained increased levels of CORT and decreased levels of T for up to seven weeks. However, besides the effect of temperature between the groups, there was also an overall decrease of CORT and T in both groups throughout the experiment. This was likely an effect of prolonged captivity on the HPI and HPG axes. Research has shown that the effects of captivity stress on animals can be highly species-specific, but decreases in both CORT and T have previously been reported in other species, including amphibians (Titon et al., 2018, Fischer & Romero, 2019). Our study was the first to report an effect of prolonged captivity on endocrine variables of *L. catesbeianus*. The impact of temperature was highest after 24 days of exposure and decreased after 47 days, therefore we cannot discard the hypothesis that these animals would have acclimated to these temperatures given enough time. Indeed, research has shown that ectotherm acclimation to different temperatures can take up to several months to achieve an asymptote depending on the species (Pintor et al., 2016), and this remains to be investigated in the American bullfrog. However, even if animals manage to acclimate, maintaining a stress response for long periods of time can dampen their ability to respond to secondary stressors and drastically impact survival in a natural setting (Romero et al., 2009), and this has been demonstrated when food acquisition is limited (Romero, 2012). Adding to that, the unintended effect of nutritional stress arising from differences in eating behaviour interacted with thermal stress in our experiment. Animals that were force fed had lower levels of T as well as higher loss of BI throughout the experiment, and these effects were more prominent when animals were also thermally stressed. Additionally, loss of BI was also correlated with hormonal stress response at day 24, with animals that had higher CORT and lower T losing more mass. In marine iguanas (*Amblyrhynchus cristatus*), body condition, associated with increased CORT levels following an *El Niño*, significantly predicted survival (Romero & Wikelski, 2001). Furthermore, in a subset of our animals, it was demonstrated that bullfrogs kept at higher temperatures also failed to show acclimation of immune variables and were significantly immunosuppressed (Lima et al., 2020). Put together, chronically elevated temperatures might negatively impact bullfrog reproduction, increase risk of disease, and lower survival; especially in conditions of food shortage, warranting further investigation.

We found significant evidence for consistent individual differences in behaviour of adult bullfrogs. Adult bullfrogs were consistent both in exploratory (AD, PC, WM) as well as risk-taking (TC) behaviour. Our repeatability estimates are congruent with values commonly reported in personality literature for other animals (Bell et al., 2009). To date, personality in the American bullfrog had only been reported in two tadpole studies, ours being the first to report significant differences in behaviour in adult *L. catesbeianus*. One study found that tadpoles did not show consistent differences in habitat choice, but did show consistent differences in their activity level (Smith & Doupnik, 2005). A different study found significant evidence for personality differences in exploration of bullfrog tadpoles, but failed to find personality in activity and boldness (Carlson & Langkilde, 2013). Considering the importance of the American bullfrog as an invasive species, knowledge of significant differences in exploration and boldness of adults could be particularly important to assist in management and conservation efforts. Studies in Australia and Hawaii investigating personality differences in the cane toad (*Rhinella marina*), another invasive anuran species, have shown that personality differences in boldness and exploration are associated with dispersion and colonization of new habitats (Gruber et al., 2017a; Gruber et al., 2017b; Gruber et al., 2018). Similar relationships have been demonstrated outside of anurans. In mosquitofish (*Gambusia sp.*), it was found that invasive species were more dispersive in comparison with native species of mosquitofish, but boldness did not consistently predict invasiveness (Rehage & Sih, 2004). Instead, differences in foraging/feeding and social behaviour predicted invasive dispersion, with invaders being more asocial and voracious (Rehage et al., 2005a; Rehage et al., 2005b; Cote et al., 2010a). However, boldness differences at the population level did impact dispersion, with individuals being more dispersive if the population was bolder and more asocial on average (Cote et al., 2011). Likewise, invasive signal crayfish (*Pacifastacus leniusculus*) were bolder, more aggressive, and more voracious than native species, but this was only true when comparing allopatric populations (Pintor et al., 2008), and authors suggest an overall aggression syndrome shaping invasiveness in this species (Pintor et al., 2008; Pintor et al., 2009). Therefore, we suggest that future studies on invasive species, such as the American bullfrog, interested in investigating how personality interacts with invasiveness should aim at exploring differences in personality from wild caught animals both in their native and invaded habitats, and highlight the importance of exploring different personality axes.

We did not find evidence to support the existence of an exploration-boldness behavioural syndrome in this species. Although exploratory variables were highly correlated between each other, neither of the three variables had a significant correlation with time spent on centre of the arena (boldness). Although this correlation is commonly found in the literature for coping styles (Koolhaas et al., 1999), several other studies have failed to find evidence for an exploration-boldness syndrome, including in amphibians, or found that syndromes were context dependent (Coleman & Wilson, 1998; D'Eath & Burn, 2002; Scales et al., 2011; Brodin et al., 2013; Kelleher et al., 2017). Evolutionary research posits that behavioural syndromes could result in maladaptive behaviour in a context-dependent manner (Sih et al., 2004a; Sih et al., 2004b). Therefore, it could be expected for behaviours to be decoupled if environmental pressures are pulling exploration and boldness in different directions, thus favouring plasticity. A recent study on convict cichlids (*Amatitlania nigrofasciata*) has demonstrated that early life predator stress effectively disrupted the formation of a behavioural syndrome, decoupling boldness and exploration in comparison with non-stressed animals (Hope et al., 2020). Alternatively, behavioural axes could become correlated if the same environmental pressure is limiting plasticity via genotype by environment interactions throughout their life, as demonstrated in the three-spined stickleback (*Gasterosteus aculeatus*). In this species, populations with different predatory pressures differ in their strength of behavioural correlations between activity, exploration and aggressiveness (Dingemanse et al., 2007), and it has been experimentally demonstrated that predator pressure was necessary for a boldness-aggression syndrome to appear (Bell & Sih, 2007). Similarly, in amphibians, a study on *Rana damaltina* tadpoles investigating the emergence of personality found that early life environmental cues were important in the development of behavioural consistency. Frogs of this species only developed personality differences if exposed to predatory cues, and only developed an activity-boldness syndrome when exposed to both predatory and social cues (Urszán et al., 2015b). Given that our animals came from frog farms where they are reared in a controlled environment, it is possible that the absence of selective pressure from predators during early stages of ontogeny could have resulted in a decoupling of exploration and boldness. Further research should focus on wild animals from a variety of contexts in order to elucidate possible mechanisms underlying behavioural syndromes and make more accurate predictions of how they might shape animal responses to environmental stress.

Furthermore, our hypothesis that personality would remain consistent in different contexts was only partially supported. While most variables showed significant correlation (PC, WM, TC) in the control group when behavioural assays were performed approximately two months apart, we found evidence to suggest that thermal stress in the experimental group weakened long-term consistency in exploratory variables and reinforced it in boldness. Our results indicate differences in plasticity and reaction norms of different behavioural variables. It is possible that when faced with environmental challenges, changes in exploratory behaviour could be beneficial. On average individuals exposed to thermal stress had a negative slope, indicating a general shift towards slower, more thorough, exploratory personality. On the other hand, reinforcement of past risk-taking behaviour by thermal stress could aid an individual to survive in a new environment where they still lack information about possible risks. It remains to be tested if this modulation of behavioural consistency by temperature confers any fitness advantages, but this shows the importance of carefully looking at different behavioural axes and their ecological significance in the context of climate change. Research on other ectotherms have also demonstrated modulation of personality variables by temperature. In coral reef fish (*Pomacentrus sp.*), personality estimates significantly increased with increased temperature (Biro et al., 2010). In hermit crabs (*Pagurus bernhardus*), the effect of high temperature depended if animals were transferred from low-to-high or from high-to-low temperatures (Briffa et al., 2013). In rock crabs *(Ozius truncates*), personality was consistent in any given temperature, but not across different temperatures (Gribben et al., 2013). Finally, in red swamp crayfish (*Procambarus clarkii*), activity and aggressiveness increased with temperature, while boldness decreased (Zhao & Feng, 2015). A proposed hypothesis for explaining this modulation by temperature is that animals should exhibit underlying individual differences in metabolic rate (Réale et al., 2010), which is known to affect behaviour of ectotherms as a function of temperature (Abram et al., 2017). These differences in behaviour and metabolism would then form a ‘pace-of-life syndrome’ (POLS), where animals with higher metabolism and bolder personality develop and grow faster (Réale et al., 2010). Given how temperature drastically affects the metabolism of ectotherms, POLS should be carefully examined as a function of temperature in these animals in the context of increasing global temperatures.

Our study was the first to investigate the relationship between personality and hormonal variables in an amphibian species. We found partial support for our hypothesis.

Bolder animals had lower levels of CORT, but only on samples collected 12 hours and 24 days after stress. However, this correlation did not emerge as an effect of thermal stress since both groups showed similar patterns. A possible explanation is that this effect could have emerged due to prolonged captivity stress, which might have been attenuated on samples after 47 days. Given that bolder animals are more exposed to predation risk and that predator encounters are potent stressors for wild animals (Narayan et al., 2013), a lower HPI reactivity in bolder animals could represent an adaptive trait, dampening the deleterious effects associated with repeated stress (Romero et al., 2009). This interpretation is supported by a study in the dark-eyed junco (*Junco hyemalis*) which found that animals from a recently colonized urban environment, which constitute a more stressful environment, were significantly bolder and had lower CORT responses in comparison with animals from the species native range (Atwell et al., 2012). Additionally, variation in T levels was consistently higher in bolder animals in all samples collected. The literature on both humans and non-human animals supports this finding (Raynaud & Schradin, 2014; Apicella et al., 2015). Particularly in frogs, increased T is associated with vocal reproductive behaviour (Marler & Ryan, 1996; Solís & Penna, 1997; Walkowski et al., 2019) and is increased during the reproductive season (Boyd, 1992; Narayan et al., 2010). We also note that our experiments were performed during the reproductive season (Medeiros et al., 2016) and that calling could be heard from bullfrogs inside climatic chambers, although we could not pinpoint which individuals were calling (*personal observation*). Calling is an energetically costly behaviour which puts individuals in higher risk of predation (Ryan et al., 1981; Tuttle & Ryan, 1981). Therefore, the positive correlation between T and boldness could be evidencing an underlying aspect of anuran reproduction in a natural setting. However, we note that further studies relating T and boldness as a function of calling/reproductive behaviours are necessary to confirm this hypothesis. Together, the results found for boldness mostly reflect what has been described in coping styles literature for mammals and birds, with bolder animals having lower levels of CORT and higher levels of T (Koolhaas et al., 1999).

Regarding exploratory behaviour, relationship with CORT and T was highly dependent on plasma samples (baseline, 12h, 24d, 47d), behavioural variable (AD, PC, WM), and experimental group (CT, IT). A study in great tits (*Parus major*) showed that CORT was positively correlated with exploration in lines specifically selected for differences in personality (fast-bold vs. slow-shy explorers), but not in unselected animals

(Baugh et al., 2012). Since our bullfrogs come from a frog farm where they developed in a controlled environment which is mostly devoid of selective pressures, a similar phenomenon might explain why we did not find a consistent relationship among different exploratory behaviours and plasma samples. It is possible that a strong relationship between CORT and exploration in this species could arise only in the presence of strong selective pressures, and this possibility should be considered for future studies with animals from wild populations. Nevertheless, there was evidence to partially support our hypothesis. We found positive correlations between exploration and CORT, with an interaction effect between group and WM; as well as a negative correlation between exploration and T. These relationships were in the opposite direction from what we predicted based on coping styles literature, with faster explorers generally having lower CORT and higher T (Koolhaas et al., 1999). The positive correlation between exploration and CORT could be related to a more general phenomenon of higher CORT being associated with increased locomotor and exploratory activity (Veldhuis et al., 1982; Landys et al., 2006), which in our experiment could translate into bullfrogs being more active inside the maze. Also, similar to our findings, in lines of *P. major* selected for personality differences slow-explorers also had higher plasma levels of T (van Oers et al., 2011). The fact that our animals did not have a boldness-exploration syndrome might explain why our findings differ from prior studies on coping styles. When different behavioural axes covary in a syndrome (e.g.: due to pleiotropy or linkage disequilibrium), it would be expected that hormonal variables would correlate with different behaviours in similar ways, even if it results in maladaptive responses depending on the context (Sih et al., 2004a; Sih et al., 2004b). However, since variation in boldness and exploration was decoupled in our animals, this might have allowed for different behaviours to vary in different directions in relation to physiological variables, which could be adaptive. In the context of reproduction, faster exploration could aid males with lower T in having more access to females, characterizing a trade-off between different coping strategies.

In this study, we have found evidence that maintenance under chronically elevated temperatures constituted a potent stressor in American bullfrogs, and that shyer animals could be more vulnerable to high environmental temperatures in the contest of climate change. Shyer animals had overall lower levels of T and higher levels of CORT and this was associated with higher loss of body condition, particularly when animals were both thermally and nutritionally stressed. This is the opposite to what is predicted for birds, with authors suggesting that proactive animals (bolder and faster explorers) would be expected to be more vulnerable (Cockrem, 2013). However, two studies on different species of fish demonstrated that personality is associated with individual thermal preference when fishes are exposed to temperature gradients. Proactive animals significantly chose higher temperatures in comparison with reactive fish (Rey et al., 2015, Cerqueira et al., 2016). Therefore, for these species of fish and similar to our results, climate change could significantly favour bolder animals in the event that individuals are unable to find colder microclimates. The relationship between personality and physiology, as well as how personality might modulate response to climate change, is still notably understudied. A lot more research is needed, particularly in species which are listed as vulnerable, before we can draw possible conclusions. Nevertheless, our study was a first step in investigating these relationships in an amphibian species and we raise attention to the fact that studies on endotherms regarding personality responses to temperature and climate change should not be readily translated to ectotherms without experimental evidence. Interestingly, the unintended effect of nutritional stress in our experiment showed interesting patterns regarding interaction with thermal stress and body condition, which warrants further investigation in studies experimentally controlling for differences in body condition or food availability. Stress literature shows that even if animals can successfully cope with one stressful situation, their response to secondary stressors can be limited (Romero et al., 2009). Furthermore, it is well known that climate change impacts on animals acts synergistically with other environmental factors such as habitat fragmentation, invasive species, pollution, and disease (Russell et al., 2009; Clark et al., 2011; Strifling, 2011; Cabral et al., 2019). Therefore, studies controlling for the effects of combined treatments should prove particularly important to aid in management and conservation efforts.

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3. Conclusões e considerações finais

Temperaturas elevadas para valores próximos da temperatura voluntária máxima de *L. catesbeianus* por 47 dias constituiu um potente estressor crônico para esses animais, os quais não apresentaram sinais de aclimatação ou habituação ao longo do experimento e mantiveram valores mais elevados de CORT e mais baixos de T ao longo de todo o experimento em comparação com animais não expostos ao estresse térmico. Notadamente, o efeito não intencional de estresse nutricional interagiu com o estresse térmico, potencializando seus efeitos e chamando a atenção para a necessidade de se investigar a interação entre diferentes estressores em estudos interessados nos possíveis impactos das mudanças ambientais sobre os animais. De forma geral, esses dados indicam que temperaturas elevadas cronicamente impactam negativamente essa espécie. Porém, é importante levar em consideração a capacidade desses animais de termorregularem comportamentalmente em condições naturais, sendo necessários estudos em campo ou estudos em laboratório que simulem diferentes microclimas para que possamos entender o real impacto que as mudanças climáticas teriam sobre esses animais.

Em relação à personalidade, os animais apresentaram alta consistência interindividual em todas as variáveis comportamentais análisadas, porém não houve indícios de síndromes comportamentais entre os eixos de exploração e boldness. Além disso, a personalidade se mostrou responsiva a mudanças de temperatura, havendo uma dimininuição da consistência a longo prazo de variáveis exploratórias e um aumento da consistência a longo prazo para boldness. Esses resultados indicam que as mudanças climáticas poderiam significativamente alterar a proporção da expressão de diferentes personalidades em populações de rãs-touro, sendo necessários mais estudos a longo prazo para avaliar essa dinâmica. Esses resultados também indicam que exploração e boldness poderiam estar sob pressões de seleção distintas nos nossos animais. Porém, vale ressaltar que estudos em outras espécies sugerem que as síndromes comportamentais se desenvolvem em função de experiências prévias com pressões ambientais e é possível que o fato dos animais utilizados não serem de vida livre tenha influenciado os resultados observados. Estudos futuros investigando a personalidade de *L. catesbeianus* em ambiente natural, tanto em populações nativas quanto invasoras, deve auxiliar em possíveis estratégias de manejo.

Finalmente, os resultados encontrados indicam que diferenças hormonais da resposta do estresse podem representar um possível mecanismo proximal para o surgimento de diferenças de personalidade nessa espécie. Animais com maiores concentrações de CORT e menores concentrações de T exploraram seu ambiente mais rápido e foram mais tímidos. Com base na literatura, esperava-se que esse perfil hormonal correspondesse a exploradores mais lentos. Porém, é possível que ausência de uma síndrome comportamental entre boldness e exploração explique essa divergência. A ausência de correlação entre os diferentes eixos comportamentais, permite que os dois eixos variem de forma diferente em função das diferenças hormonais, o que poderia representar uma resposta adaptativa. Finalmente, o fato dos animais mais tímidos terem apresentado maiores concentrações de CORT, menores concentrações de T e também uma maior perda de condição corpórea ao longo do experimento poderia impactar sua capacidade reprodutiva e sobrevivência na natureza. Dessa forma, esses resultados indicam que animais mais tímidos estariam mais vulneráveis às mudanças climáticas, o que é de grande relevância para futuros estudos de conservação em anfíbios em diferentes contextos associados a mudanças ambientais antropogênicas.