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Vanessa Andreatta de Quadros

AVALIAÇÃO DO PADRÃO COMPORTAMENTAL DE DUAS LINHAGENS DE PEIXE ZEBRA EXPOSTAS À SUBSTÂNCIA DE ALARME EM DIFERENTES CONTEXTOS

Santa Maria, RS
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Dissertação apresentada ao Curso de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requerido parcial para obtenção do título de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

Orientador: Prof. Dr. Denis Broock Rosemberg

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Aprovado em 25 de julho de 2016:

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Santa Maria, RS
2016

DEDICATÓRIA

Dedico este trabalho às pessoas mais presentes na minha vida:

Primeiramente a Deus, pela força e coragem para enfrentar os obstáculos desta caminhada.

A minha mãe, pelo exemplo de coragem e amor.

Ao meu pai, o mais generoso e companheiro de todos os pais.

A minha irmã, Fernanda, pelas palavras de incentivo.

Meus maiores PRESENTES SÃO VOCÊS

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Na vida tudo se resume em três coisas, INTERESSE, DISPOSIÇÃO e TEMPO. Se você não possuir disposição para encontrar tempo é porque nunca existiu real interesse.

(Autor desconhecido)

RESUMO

AVALIAÇÃO DO PADRÃO COMPORTAMENTAL DE DUAS LINHAGENS DE PEIXE ZEBRA EXPOSTAS À SUBSTÂNCIA DE ALARME EM DIFERENTES CONTEXTOS

AUTORA: VANESSA ANDREATTA DE QUADROS
ORIENTADOR: DENIS BROOCK ROSEMBERG

O peixe zebra (*Danio rerio*) é um pequeno teleósteo amplamente utilizado em pesquisas científicas nas diferentes áreas do conhecimento. Dentre as características que tornam o peixe zebra um atrativo organismo modelo quando comparado com roedores podemos citar a facilidade de manutenção, a manipulação e a viabilidade para a avaliação de aspectos neuroquímicos que influenciam o comportamento animal. O genoma do peixe zebra já foi totalmente sequenciado e sua alta homologia com genes humanos possibilita a utilização da espécie em estudos translacionais de estresse e comportamento defensivo. Em peixes, a exposição à substância de alarme de co-específicos é um modelo experimental capaz de induzir medo, mimetizando de modo natural a comunicação química que ocorre em situações de ataque de predadores. Na literatura, a análise comportamental de duas linhagens de peixe zebra (selvagem "*WT*" e leopardo "*leo*") demonstrou que *leo* apresenta uma exacerbação do comportamento tipo ansiedade, o que poderia ser um importante aspecto para a investigação dos comportamentos defensivos desencadeados pela substância de alarme em diferentes situações experimentais. Assim, o objetivo deste trabalho foi avaliar o padrão comportamental das linhagens de peixe zebra *WT* e *leo* expostas agudamente à substância de alarme (5 min) em três contextos: durante o período de exposição (Experimento 1); após a exposição, em respostas de habituação à novidade (Experimento 2); ou após a exposição, na preferência claro-escuro (Experimento 3). Além disso, verificamos a influência da substância de alarme sobre a resposta pigmentar de ambas as linhagens. Durante a exposição à substância de alarme (Experimento 1), a linhagem *leo* aumentou o nado vertical, bem como o número e a duração de movimentos erráticos, enquanto que *WT* aumentou o número de movimentos erráticos e a latência para entrada na área superior do aparato. Ambas as linhagens não apresentaram variação no padrão de pigmentação. No teste do tanque novo (Experimento 2), a linhagem WT apresentou mais respostas relacionadas ao medo. Por outro lado, *leo* apresentou um aumento das transições e do tempo gasto no topo, indicando diferenças na habituação. A substância de alarme aumentou o número de movimentos erráticos no teste claro-escuro (Experimento 3), mas desencadeou diferentes respostas na escototaxia, na latência para entrada no compartimento escuro e nos episódios de avaliação de risco. A análise principal de componentes sugeriu que o nado acelerado, comportamentos tipo ansiedade e locomoção/exploração foram os fatores que mais contribuíram para os resultados obtidos nos Experimentos 1, 2 e 3, respectivamente. Em suma, a substância de alarme apresenta uma resposta dependente de linhagem e contexto em peixe zebra.

Palavras chave: Medo, ansiedade, comportamentos defensivos, peixe zebra.

ABSTRACT

EVALUATION OF THE BEHAVIORAL PROFILE OF TWO ZEBRAFISH STRAINS EXPOSED TO ALARM SUBSTANCE AT DIFFERENT CONTEXTS

AUTHOR: VANESSA ANDREATTA DE QUADROS
ADVISOR: DENIS BROOCK ROSEMBERG

The zebrafish (*Danio rerio*) is a small teleost widely used in several scientific researches. Among the characteristics that make zebrafish an attractive model organism when compared to rodents, we highlight the easy maintenance, manipulation, and the feasibility for the evaluation of neurochemical aspects that influence animal behavior. The zebrafish genes have been already sequenced and present a high degree of homology in relation to their human counterparts, which allows the use of this species in translational studies of stress and defensive behavior. In fish, exposure to alarm substance from conspecifics is an experimental model that induces fear, naturally mimicking the chemical communication that occur during the attack of predators. In the literature, the behavioral analysis of two zebrafish strains (*wild type "WT"* and *leopard "leo"*) showed that *leo* presents an exacerbation of anxiety-like behaviors. This aspect could be an important characteristic for assessing the defensive behaviors triggered by alarm substance in distinct experimental approaches. Thus, the goal of this work was to evaluate the behavioral profile of *WT* and *leo* acutely exposed to alarm substance from conspecifics (5 min) at three contexts: during the alarm substance exposure period (Experiment 1); after exposure, in habituation to novelty (Experiment 2); or after exposure, in the light-dark test (Experiment 3). Furthermore, we verified the influence of alarm substance on pigment response. During the alarm substance exposure (Experiment 1), *leo* increased the vertical drifts, as well as the number and duration of erratic movements, while *WT* increased the number of erratic movements and the latency to enter the upper area of the apparatus. Both strains did not present significant changes on pigment response. In the novel tank test (Experiment 2), *WT* presented increased fear responses. On the other hand, *leo* increased the vertical exploration, indicating differences on habituation to novelty. In the light-dark test (Experiment 3), the alarm substance promoted a significant increase in the number of erratic movements, but elicited different responses in scototaxis, in the latency to enter the dark compartment, and in the number of risk assessment episodes. Principal component analyses suggested that burst swimming, anxiety-like behaviors, and locomotion/exploration were the main factors that contributed to the results obtained in the Experiments 1, 2, and 3, respectively. Altogether, the behavioral responses of alarm substance from conspecifics in zebrafish are strain- and context-dependent.

Keywords: Fear, anxiety, defensive behaviors, zebrafish.

LISTA DE ABREVIATURAS

AC – Amigdala central

cx41.8 – Conexina 41.8

HHI – Eixo Hipotálamo-hipófise-inter-renal

HM – Habênula medial

leo – Linhagem leopardo

NLET – Núcleo do leito da estria terminal

ZBC – Catálogo comportamental do peixe zebra (do inglês, *Zebrafish Behavioral Catalogue*)

WT – Linhagem selvage (do inglês, *wild type*)

SUMÁRIO

1. APRESENTAÇÃO

Esta Dissertação aborda assuntos relacionados aos efeitos mediados pela substância de alarme no comportamento defensivo em vertebrados, utilizando o peixe zebra como organismo modelo. Ela encontra-se estruturada da seguinte forma:

INTRODUÇÃO: Revisão da literatura com caracterização dos temas abordados na dissertação.

MATERIAIS E MÉTODOS, RESULTADOS e DISCUSSÃO: Serão apresentados na forma de artigo científico.

CONCLUSÃO: Comentários gerais sobre os resultados obtidos no trabalho.

PESPECTIVAS: Apresentação das possibilidades de novos estudos a partir dos resultados obtidos.

REFERÊNCIAS: Lista as referências utilizadas na introdução da Dissertação.

2. INTRODUÇÃO

2.1. Medo e ansiedade

Diversos comportamentos defensivos fazem com que os animais tenham um sistema de "auto-proteção". Esses comportamentos são semelhantes aos repertórios apresentados pelos mamíferos quando analisados em contextos de situações específicas aversivas, desencadeando respostas de medo e ansiedade (Blanchard et al. 1989; Blanchard et al. 1993a). Mesmo utilizados como sinônimos, medo e ansiedade possuem significados distintos, onde a ansiedade se difere do medo em base na proximidade de estímulos e certeza. Nesse sentido, o medo é uma resposta imediata ou uma ameaça iminente, enquanto que a ansiedade é caracterizada como uma resposta de ameaça potencial ou distante (Blanchard et al. 1989; Maximino et al. 2010a). Dessa maneira, estudos vem sendo desenvolvidos com o propósito de verificar os aspectos comportamentais e neuroquímicos envolvidos nas respostas de medo e ansiedade em diversas espécies (Jesuthasan 2012).

Em humanos, somente nos Estados Unidos da América (EUA), os transtornos de ansiedade atingem aproximadamente 40 milhões de adultos e o tratamento pode custar mais de 42 bilhões de dólares por ano (Hurrell et al. 2016). Respostas exacerbadas de medo ou ansiedade estão relacionadas com eventos de fobia social, pânico, estresse pós traumático, transtorno obsessivo-compulsivo, e até mesmo a depressão (Cattell 1966; Davis et al. 2010; Jesuthasan 2012). Assim, o estudo dos aspectos emocionais associados a fatores ambientais e genéticos poderia ser útil para a compreensão dos fenótipos e dos mecanismos bioquímicos envolvidos em transtornos neuropsiquiátricos, bem como possibilitar a busca por intervenções farmacológicas a fim de obter tratamentos efetivos (Adolphs et al. 1995; Crawley 1989; Kalueff et al. 2007; Kalueff & Tuohimaa 2004; Maximino et al. 2013; Rodgers et al. 1997).

Estudos sugerem que fenótipos associados ao medo e ansiedade em modelos experimentais podem ser diferenciados farmacologicamente. Por exemplo, a administração de benzodiazepínicos e citalopram responde para o tratamento de ansiedade, mas não em modelos de medo (Howland 2016). Outro aspecto importante é que ambas as respostas podem envolver diferentes estruturas cerebrais (Blanchard et al. 1993b; Davis & Waters 1997; Grillon et al. 2009; Maximino et al. 2013; McNaughton & Gray 2000). É sabido que a habênula medial (HM) e amígdala são responsáveis por regular respostas de medo. Lesões causadas no centro da amígdala reduz o comportamento associado com o medo, indicando que esta região encontra-se ativada nessas condições (Agetsuma et al. 2010; Blanchard & Blanchard 1972; Kapp et al. 1979). Outra estrutura importante que recebe informações do

centro da amigdala é o núcleo do leito da estria terminal (NLET), a qual aparentemente não está correlacionada com respostas de medo, mas possui um importante papel nas respostas de ansiedade (Hitchcock & Davis 1991; Davis et al. 2010). Estudos em seres humanos já foram realizados com o objetivo de entender os processos moleculares e as bases neuroquímicas associadas à evolução de diferentes transtornos neuropsiquiátricos. No entanto, as complicações envolvidas em pesquisas com humanos, tanto em aspectos éticos quanto operacionais, refletem a necessidade de se investir em pesquisas básicas utilizando espécies de laboratório (Jesuthasan 2012).

Até o presente momento, embora os estudos dos comportamentos relacionados ao medo e ansiedade em roedores sejam amplos, estes apresentam um elevado custo e possuem um baixo rendimento para triagens de novos fármacos (Leung & Mourrain 2016). Considerando que o sistema neurológico dos vertebrados foi altamente conservado durante a evolução, a análise dos mecanismos envolvidos em respostas de medo e ansiedade em modelos animais alternativos pode ter grande relevância para uma melhor compreensão dessas emoções (Jesuthasan 2012).

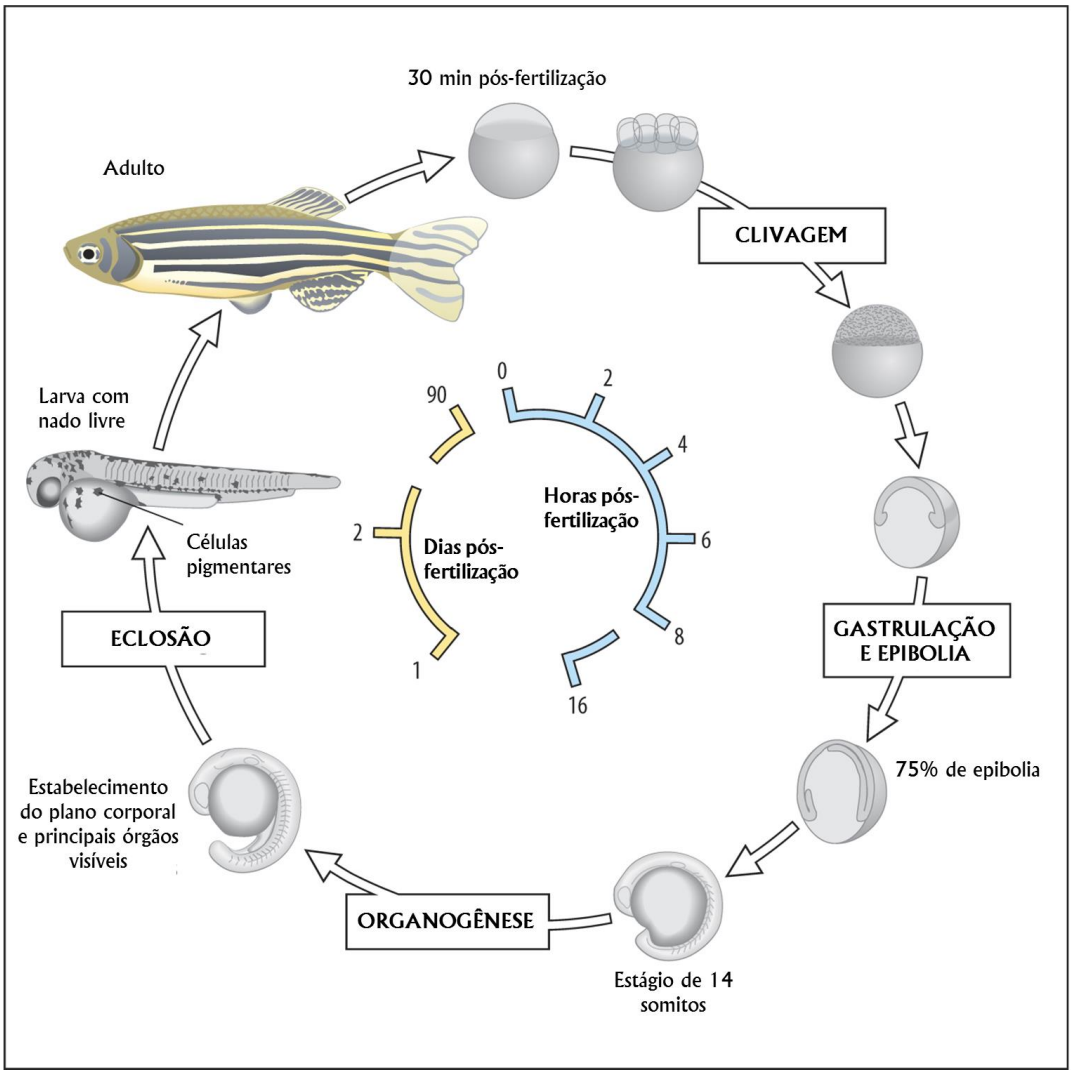
2.2. A utilização do peixe zebra na pesquisa científica

O peixe zebra (*Danio rerio*) é uma espécie tropical de água doce conhecida popularmente como “paulistinha”, o qual pertence à família Cyprinidae e é nativo da Ásia (Whitlock & Westerfield 2000). Dentre as vantagens apresentadas podemos citar o pequeno tamanho (adultos podem medir de 3 a 5 cm), o baixo custo e o pequeno espaço requerido para manutenção, a grande prole (50 a 200 ovos por dia para cada fêmea em condições otimizadas de reprodução), a presença de ovos translúcidos e o rápido desenvolvimento até a fase adulta (aproximadamente 2–3 meses) (Dahm & Geisler 2006; Lele & Krone 1996) **(Figura 1)**.

O primeiro pesquisador a estudar a biologia do peixe zebra no final da década de 60 foi George Streisinger, que utilizou técnicas de mutagênese sítio dirigidas para estudos relacionados à biologia do desenvolvimento (Dahm & Geisler 2006; Grunwald & Eisen 2002; Gulati-Leekha & Goldman 2006; Rinkwitz et al. 2015). O genoma do peixe zebra já é completamente sequenciado e seus genes possuem um alto grau de homologia com os genes dos mamíferos, o que possibilita modelar doenças humanas (Howe et al. 2013). Devido a esses aspectos, o peixe zebra combina a relevância de ser um vertebrado na escala de invertebrado, o que favorece a elaboração de protocolos de triagens de médio/alto rendimento

quando comparado a roedores em estágios pré-clínicos (Goldsmith 2004; Leung & Mourrain 2016) **(Figura 2)**.

Figura 1 – Desenho esquemático das etapas do desenvolvimento do peixe zebra.



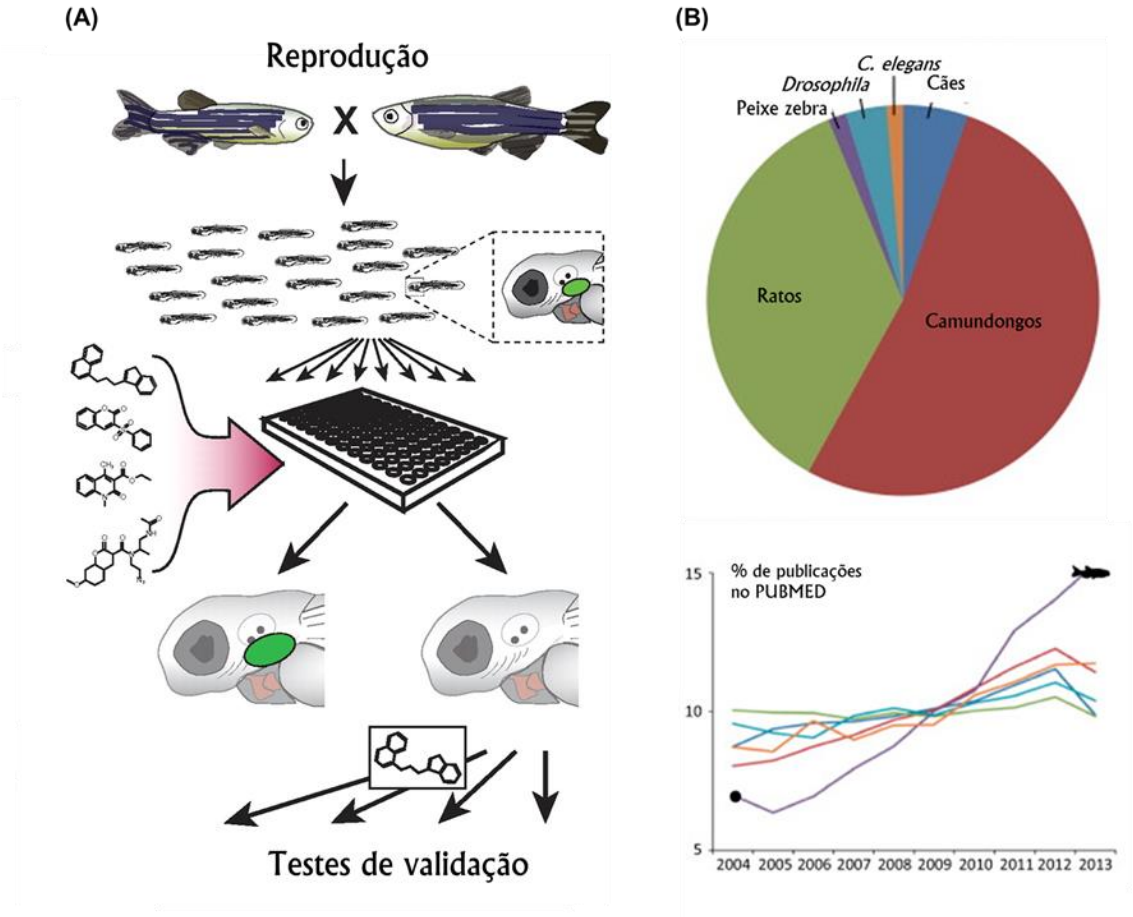
Fonte: http://www.mun.ca/biology/desmid/brian/BIOL3530/DEVO_03/ch03f09.jpg (adaptado e acessado em julho de 2016).

Estudos utilizando o peixe zebra vem sendo desenvolvidos em áreas como bioquímica, neuroquímica, e farmacologia e biologia do comportamento (Blaser et al. 2010; Edwards & Michel 2002; Egan et al. 2009; Fontana et al. 2016; Gerlai 2003; Maximino et al. 2011; Mezzomo et al. 2016; Piato et al. 2011; Rosemberg et al. 2011).

Assim, o uso do peixe zebra está crescendo gradativamente como um modelo animal potencialmente útil para estudos dos comportamentos defensivos e para a investigação de

fenótipos relacionados ao medo e à ansiedade (Cachat et al. 2010; Gerlai 2010, 2011; Maximino et al. 2010a; Stewart et al. 2011).

Figura 2 – Uso do peixe zebra na pesquisa científica. **(A)** Aplicação em testes de triagem de compostos em fase pré-clínica. **(B)** Diagramas representativos do número de publicações utilizando a espécie no Pubmed.



Fonte: Adaptado de Clements & Traver, 2012 e Stewart et al., 2014.

2.3. Estudos sobre medo e ansiedade em peixe zebra

As respostas de medo e ansiedade são diferenciadas conforme a proximidade ou a certeza do perigo, respectivamente. Estudos relacionados com modelos que induzem comportamentos do tipo medo e ansiedade em peixe zebra ainda não são totalmente elucidados. No entanto, diversos trabalhos tem mostrado avanços significativos nesta área do conhecimento (Fanselow 1994; Guo 2009; Kokel & Peterson 2008; Maximino et al. 2013).

Atualmente, muitas pesquisas neuroquímicas, neuroanatomicas e farmacológicas em peixe zebra estão sendo desenvolvidas a fim de buscar novos conhecimentos das emoções, como medo e ansiedade. (Guo et al. 2012; Jesuthasan 2012). Nesse contexto, sabe-se que respostas a agentes estressores apresentada pelo peixe zebra estão relacionadas ao eixo hipotálamo-hipófise-interrenal (HHI), assim como, o hormônio cortisol, envolvido na resposta ao estresse, o qual aumenta significativamente após a exposição a fatores estressores (Dal Santo et al. 2014; Oliveira et al. 2014). Além disso, o encéfalo possui regiões específicas caracterizadas por respostas a estímulos aversivos, como a amígdala central (AC), responsável em respostas de ameaça de medo ou resposta imediata, cujas lesões pode causar redução ou perda do comportamento associada ao medo, e a região da habenula, ligada as respostas de ameaça potencial, comportamento emocional e expressão da memória (Amo et al. 2010; Blanchard & Blanchard 1972; Kapp et al. 1979; Maximino et al. 2013; McNaughton & Gray 2000; Okamoto et al. 2012). No entanto, a forma na qual o encéfalo determina essas ameaças, identificando as regiões específicas para respostas de medo e ansiedade, ainda são desconhecidas (Oliveira et al. 2013).

Dessa forma, a utilização do peixe zebra como modelo animal vem sendo cada vez mais desenvolvidas em estudos de laboratório, com a finalidade de entender e compreender os mecanismos envolvidos nestas emoções (Maximino et al. 2013; Speedie & Gerlai 2008).

2.4. Testes comportamentais em peixe zebra

O uso peixe zebra vem ganhando espaço nas áreas de pesquisa em neurociência, com aplicabilidade para a compreensão do repertório comportamental em pesquisas de cunho ecológico e na medicina translacional (Leung & Mourrain 2016). A compreensão dos mecanismos neurológicos e dos fenótipos comportamentais associados na espécie pode fornecer importantes descobertas sobre potenciais biomarcadores e sobre as bases genéticas envolvidas em diversas patologias (Rico et al. 2011). Nesse sentido, foi desenvolvido um catálogo detalhado no qual os pesquisadores identificaram e caracterizaram os tipos de comportamento existentes no peixe zebra, tanto em estágio larval quanto adulto (Zebrafish Behavioral Catalogue , ZBC) (Kalueff et al. 2013).

Diversos estímulos são comumente utilizados para a investigação de parâmetros relacionados ao medo e à ansiedade em peixe zebra. Um dos estímulos empregados é a novidade, onde o teste da exposição a novos ambientes propõe avaliar os perfis de locomoção, motricidade e exploração vertical dos animais, determinando-se o padrão de habituação

durante o teste (Egan et al. 2009; Grossman et al. 2010; Mathur et al. 2011; Maximino et al. 2010a; Rosemberg et al. 2011; Sackerman et al. 2010). Outro estímulo para verificar respostas de ansiedade é o conflito gerado na presença de ambientes aversivos e preferenciais. Em peixe zebra, a escototaxia é caracterizada pela preferência a ambientes escuros em detrimento a ambientes claros (Maximino et al. 2010a) e, nesse contexto, o teste do aquário claro-escuro tem sido validado como um modelo para a avaliação do comportamento tipo ansiedade para a espécie (Blaser et al. 2010; Maximino et al. 2011; Maximino et al. 2010a; Rosemberg et al. 2012). Portanto, estudos referentes aos comportamentos relacionados ao medo e à ansiedade em peixe zebra apresentam um grande potencial para a avaliação dos padrões comportamentais apresentados por indivíduos que possuem diferentes características genéticas e fisiológicas (Maximino et al. 2013).

2.5. Linhagens de peixe zebra: selvagem e leopardo

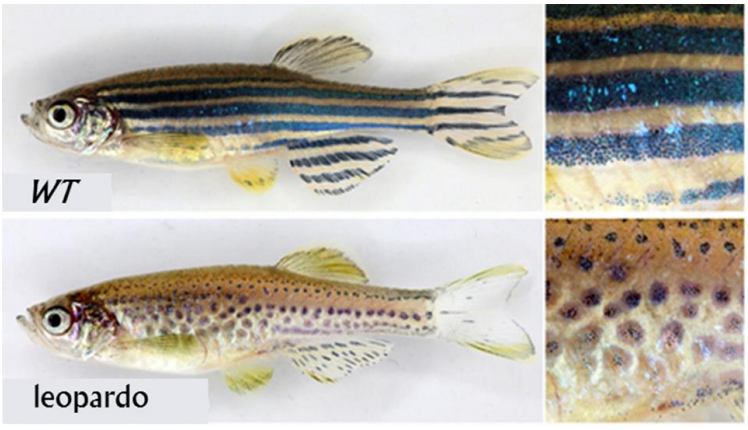
A seleção de diferentes linhagens pode ter grande aplicação no design experimental, já que diversos estudos têm comprovado que diferentes linhagens de peixe zebra possuem genética e comportamentos tipo ansiedade distintos (Egan et al. 2009; Howe et al. 2013). Assim, a seleção poderia melhorar a triagem de compostos ansiolíticos (efeitos mais detectáveis em linhagens ansiosas, como por exemplo, o *leopardo* [*leo*]) e ansiogênicos (linhagens com comportamento tipo ansiedade reduzido, como a selvagem [WT]) (Egan et al. 2009; Maximino et al. 2013) **(Figura 3)**.

As características do padrão das células de pigmentação são importantes para a determinação de linhagens do peixe zebra. O formato e a coloração completa se definem aproximadamente entre 3 a 6 semanas de desenvolvimento (Frohnhofer et al. 2013). A linhagem selvagem é caracterizada por possuir linhas alternadas de coloração clara/escura, horizontais ao longo do corpo até a cauda, enquanto a linhagem *leo* desenvolve uma série de pontos escuros não uniformes em todo o corpo (Frankel 1979; Kirschbaum 1975). Alelos dominantes foram identificados no peixe zebra e demonstrados que o fenótipo do *leo* é causado por uma mutação no gene *connexin 41.8* (*Cx41.8*) (Haffter et al. 1996; Watanabe et al. 2012), resposável pela codificação da junção de hiato (gap junction protein α5, GJA5). As junções de hiato são canais intercelulares que permitem a passagem de pequenas moléculas e íons entre células vizinhas e, portanto, importantes em funções de química e acoplamento elétrico (Irion et al. 2014). Já a linhagem *leo* é conhecida por possuir dois tipos de cromatóforos: os melanóforos e os xantóforos, com interações homotípicas e heterotípicas

(Maderspacher & Nusslein-Volhard 2003). A mutação no gene *Cx41.8* em *leo* resulta em alterações significativas no padrão de organização dos melanossomos em comparação com a *WT* (Watanabe et al. 2012).

Como uma resposta inata apresentada por diversos vertebrados, o padrão de pigmentação possui um importante papel na obtenção de alimento, na comunicação social e nas respostas anti-predatórias (Fujii et al. 2000; Nascimento et al. 2003). A modulação da resposta pigmentar pode estar associada com mudanças em parametros do comportamento tipo ansiedade, na resposta natural dos individuos expostos ao escuro e a ambientes iluminados (resposta de camuflagem), na interação social (comportamentos de acasalamento ou agressivos), bem como ser resultado de exposição a compostos químicos ou a agentes estressores (Kalueff et al. 2013; Wagle et al. 2011). A resposta pigmentar envolve uma mudança global na cor dos animais, a qual resulta em uma aparência mais clara ou escura do peixe zebra, cujos mecanismos associados ainda não são completamente elucidados (Echevarria et al. 2011; Gerlai et al. 2000; Logan et al. 2006). Assim, o estudo sobre as respostas pigmentares em linhagens distintas pode servir como uma ferramenta útil para verificar os sinais químicos que induzem medo e comportamento tipo ansiedade em diferentes contextos (Barba-Escobedo & Gould 2012; Egan et al. 2009; Moretz & McKay 2008; Wright et al. 2003).

Figura 3 – Peixes zebra das linhagens selvagem (*WT*) e leopardo (*leo*), com detalhes do padrão de pigmentação em listras azuladas ou pontos escuros dispersos.



Fonte: http://www.eb.tuebingen.mpg.de/research/emeriti/research-group-colour-pattern-formation.html (adaptado e acessado em julho de 2016).

2.6. Modelos de indução de medo em peixe zebra

Gray (1987) estudou e propôs cinco categorias de estímulos que poderiam induzir medo. A primeira categoria se refere aos estímulos predatórios, como a simples presença de um predador no tanque ou a visualização do mesmo por um grupo de peixes (Ahmed et al. 2011; Bass & Gerlai 2008; Gerlai et al. 2009; Jorgensen et al. 2012; Luca & Gerlai 2012; McHenry et al. 2009; Parra et al. 2009; Speedie & Gerlai 2008). A segunda é a novidade, onde o animal é colocado em um ambiente desconhecido e tende a habituar-se ao longo do tempo, como o observado no teste de exposição a um novo tanque (Cachat et al. 2010; Cachat et al. 2011; Levin et al. 2007; Rosemberg et al. 2011; Wong et al. 2010). A terceira categoria é o choque elétrico de baixa ou moderada intensidade, considerado um estímulo condicionado eficaz (Xu et al. 2007). A quarta caracteriza-se por estímulos intensos, como por exemplo um barulho alto, aumento de estados vibracionais moleculares ou exposições a ambientes com luz intensa. Em peixe zebra, utiliza-se o aparato claro/escuro, onde o animal tende a evitar ambientes claros (Blaser et al. 2010; Blaser & Penalosa 2011; Burgess & Granato 2007; Eaton et al. 1977; Hatta & Korn 1998; Kimmel et al. 1974; Maximino et al. 2010b; Stephenson et al. 2011). Por fim, a quinta categoria inclui a utilização de substâncias químicas aversivas (Egan et al. 2009; Gaikwad et al. 2011; Mourabit et al. 2010; Parra et al. 2009; Speedie & Gerlai 2008; Wong et al. 2010).

Dentre os repertórios de comportamentos defensivos apresentados pelos peixe zebra, o aumento dos episódios de congelamento e da frequência de movimentos erráticos são comumente associados ao medo (Egan et al. 2009; Lima et al. 2016; Speedie & Gerlai 2008). O congelamento é descrito como um episódio de imobilidade por mais de 2 segundos, onde o animal apresenta movimento dos olhos e uma alta frequência de movimento opercular. Já os movimentos erráticos podem ser definidos como mudanças abruptas na direção de nado, com aceleração de movimentos esquerda-direita (Kalueff et al. 2013). Outros comportamentos que podem ser observados em situações aversivas é a maior permanência dos animais no fundo do aquário e a menor exploração vertical (Rosemberg et al. 2012), o aumento do tempo gasto em compartimentos escuros (Lima et al. 2016; Maximino et al. 2010c) e o aglomeramento do cardume, que constitui uma unidade funcional coesa para proteção (Green et al. 2012; Schmidel et al. 2014). Apesar das cinco categorias propostas por Gray serem eficazes em induzir medo em peixe zebra, a influência de diferentes contextos nas linhagens *WT* e *leo* expostas a componentes químicos aversivos naturais para a espécie, tais como a substância de alarme, ainda não foi rigorosamente estudada.

2.7. Substância de alarme de co-específicos

Em 1938, Karl von Frisch descreveu pela primeira vez uma substância denominada *"Schreckstoff",* definida por outros autores mais tarde como substância de alarme, feromônio de alarme ou mensageiro químico de alerta (Smith, 1992). Diversas hipóteses foram postuladas para determinar os aspectos evolutivos da substância de alarme. A primeira hipótese relaciona-se ao grau de parentesco e a substância seria liberada no meio para facilitar a identificação de indivíduos próximos. A segunda proposta foi caracterizada como hipótese de atração do predador. Tal hipótese sugere que a liberação da substância de alarme serve para atrair predadores adicionais a fim de interferir na predação anterior e gerar competição pelo recurso, onde a presa teria mais chances de escape. A terceira hipótese refere-se à função imunológica dos animais, onde a substância poderia inibir o crescimento de patógenos e parasitas, além de proteger contra a radiação ultravioleta. A última hipótese refere-se à capacidade da substância de alarme atuar na proteção de lesões físicas como ocorre em eventos de ataque por predadores. No entanto, pesquisadores defendem a ação da substância de alarme como um mensageiro químico aversivo de alerta, beneficiando, assim, o cardume contra predação (Smith, 1992; Maximino et al., 2012).

A substância de alarme é produzida por células epiteliais especializadas de peixes teleósteos e liberada na água quando ocorre algum dano na pele no animal. Ela é detectada pelo sistema olfativo e exerce uma sinalização química que resulta em respostas comportamentais do tipo "luta ou fuga" dos animais (Mourabit et al. 2010). No entanto, esta substância é extremamente volátil, permanecendo no máximo 30 minutos em ação (Parra et al. 2009). No entanto, dados da literatura mostram que uma única exposição à substância de alarme é capaz de exacerbar o repertório de comportamento defensivo em peixe zebra durante 24 horas, sugerindo que o modelo poderia ser útil em estudos relacionados ao estresse pós traumático (Lima et al. 2016).

A caracterização química da substância de alarme de peixe zebra ainda não é totalmente elucidada, porém o composto químico 3-N-óxido de hipoxantina (H_3NO) foi identificado como uma das moléculas presentes nessa substância, a qual é capaz de induzir respostas aversivas de congelamento e movimentos erráticos em peixe zebra na sua forma isolada (Speedie & Gerlai 2008). Além disso, a substância de alarme é caracterizada por ser uma mistura complexa, uma vez que possui fragmentos de glicosaminiglicanos e sulfato de condroitina, capazes de induzir respostas comportamentais diferenciadas em peixe zebra. Além disso, acredita-se que o sulfato de condroitina seja a molécula responsável por ativar um

subconjunto de neurônios olfatórios (Argentini, 1976; Pfeiffer, 1982; Brown et al., 2000; Mathuru et al., 2012). Quando a substância de alarme de co-específicos é extraída da pele de um peixe zebra doador, o animal receptor exposto realiza movimentos erráticos, podendo permanecer no fundo do tanque em comportamento de congelamento ou aglomeração, ou tornar-se mais pálido, alterando seu padrão de pigmentação (Speedie & Gerlai 2008). A extração da substância de alarme é um protocolo de fácil execução e não exige recursos financeiros extremos. Além disso, é bem caracterizada por produzir comportamento do tipo medo, pois é capaz de aumentar a expressão do gene *c-fos* na habênula de peixe zebra (Nathan et al. 2015; Ogawa et al. 2014) e, dessa forma, a substância de alarme pode ser classificada na quinta categoria proposta por Gray (1987). De fato, a validação de modelos comportamentais que analisem as respostas de diferentes linhagens a mensageiros químicos naturalísticos que induzam medo poderia servir como uma importante ferramenta para investiga as causas proximais das reações de alarme em vertebrados desafiados a diferentes contextos.

3. OBJETIVOS

O objetivo da presente dissertação foi avaliar se as respostas comportamentais de peixes zebra expostos à substância de alarme são dependentes de linhagem e contexto. De modo específico, utilizando as linhagens *WT* e *leo*, os objetivos são:

- Investigar o padrão dos comportamentos relacionados ao medo durante o período de exposição à substância de alarme;
- Verificar o padrão pigmentar dos peixes após a exposição à substância de alarme;
- Analisar a influência da substância de alarme na locomoção, exploração e habituação à novidade;
- Determinar os efeitos da substância de alarme na exploração a ambientes com contexto claro/escuro;
- Avaliar quais respostas comportamentais poderiam ser responsáveis pelos efeitos da substância de alarme em diferentes contextos.

4. ARTIGO CIENTÍFICO

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Strain- and context-dependent behavioural responses of acute alarm substance exposure in zebrafish

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ABSTRACT

We investigate the behavioural responses of wild type (*WT*) and leopard (*leo*) zebrafish elicited by alarm substances of conspecifics at three contexts: during the exposure period (Experiment 1); after exposure, in habituation to novelty (Experiment 2); or after exposure, in the light–dark preference test (Experiment 3), and analyse their influence on pigment response. During the exposure, *leo* showed decreased vertical drifts, increased number and duration of erratic movements, while *WT* had increased erratic movements and latency to enter the top. In the novel tank, we observed that angular velocity decreased in *WT* exposed to alarm substance, which also presented increased fear responses. Contrastingly, *leo* increased the number of entries and time in top, indicating differences in habituation profile. Alarm substance increased the number of erratic movements in the light–dark test, but elicited different responses between strains in scototaxis, latency to enter the dark compartment and risk assessment episodes. Moreover, the body colour of zebrafish did not change after alarm substance exposure. Principal component analyses suggest that burst swimming, anxiety-like behaviours, and locomotion/exploration were the components that most accounted for total variances of Experiments 1, 2, and 3, respectively. We conclude that chemical cue from conspecifics triggers strain- and context-dependent responses.

1. Introduction

Chemical alarm cues exist in a wide range of taxa and allow individuals to interpret specific characteristics of the environment, as potential threats (Ferrari et al., 2010; Wishingrad et al., 2015). In fish species, alarm substance (also called alarm cue or Schrekstoff) may be released when skin is damaged, as occurs after a predator attack (Rehnberg et al., 1987; Oliveira et al., 2014). Alarm substance may elicit innate defensive behaviours to conspecifics after detection by specific chemoreceptors, triggering darting behaviours, also known as erratic movements, freezing and even agglomeration in the bottom of the tank (Mourabit et al., 2010; Speedie and Gerlai, 2008). Animals have the capacity to display different fear responses depending on the genetic background, environment and the threat stimulus (Stankowich and Blumstein, 2005). Indeed, the validation of behavioural models that analyse the responses of distinct strains to chemical cues that induce fear could serve as valuable tools to investigate the underlying proximate causation of alarm reactions in vertebrates when challenged to different contexts.

The zebrafish (*Danio rerio*) is a suitable organism for neurobehavioural studies due to the combination of well-characterised attributes. First, the zebrafish genome has already been characterised, presenting a high degree of homology (around 70%) with its human counterpart (Howe et al., 2013). Despite the anatomical differences, brain functions of several analogue structures are preserved and all the major neurotransmitter systems of mammals have been identified in the central nervous system (CNS) of this species (Rico et al., 2011; Stewart et al., 2014). Second, zebrafish also offer an excellent potential to behavioural studies because they present a wide range of behaviours that can be measured at several

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contexts (Blaser and Rosemberg, 2012; Egan et al., 2009; Maximino et al., 2013; Moretz et al., 2007; Rosemberg et al., 2012).

In the literature, it has been described the existence of distinct zebrafish strains. Although the genetic characterisation of the short fin wild type (*WT*) strain has not been completely understood, it is expected to be genetically heterogeneous, with an evolutionary conserved gene pool due to the proximity of its breeding facility to the geographic origin of zebrafish in Singapore (Pan et al., 2012). This phenotype is characterised by the presence of continuous blue stripes across the lateral surface of the body with decreased length of bony segments from the caudal fin (Hoptak-Solga et al., 2007). The zebrafish leopard phenotype (*leo*) displays irregular pigmentation, presenting spots instead of stripes associated to mutations in *connexin* 41.8 gene (Irion et al., 2014; Watanabe et al., 2006). Moreover, both strains present robust basal behaviour differences in which *leo* shows pronounced defensive behavioural responses, usually known as increased anxiety-like responses in translational studies (Egan et al., 2009; Maximino et al., 2013). It is conceivable that trait-anxious individuals frequently experience fear across a range of different contexts and threats (Grupe and Nitschke, 2013). In this regard, the involvement of genetic factors in alarm substance-mediated responses and their relationship with different contexts, such as habituation to novelty and light–dark preference are still obscure.

In the current report, we sought to investigate whether the behavioural responses triggered by alarm substance are strain- and context-dependent in zebrafish. The chemical cues were extracted from *WT* and *leo* and the actions were further tested in their respective conspecifics. We used three approaches in order to analyse the behavioural patterns: during the exposure period (Experiment 1—observation cylinder test); after exposure, in habituation to novel environments (Experiment 2—novel tank test); or after exposure, when experienced to dark-bright contexts (Experiment 3—light–dark test). To verify whether the alarm substance can affect the colour of zebrafish, a variable that can be modulated by stressor agents, we also determine its influence on pigment response.

2. Materials and methods

2.1. Animals

Adult zebrafish (*Danio rerio*) of short fin wild type (*WT*) and leopard (*leo*) strains were purchased from the same local commercial distributor (Hobby Aquários, RS, Brazil). Animals were 4–6 months-old and a 50:50 male:female ratio was used for the experiments. Phenotypically, *leo* spot pattern was similar to t1/t1 variant described previously (Watanabe et al., 2006). Fish were raised in 40 L tanks separated by pigment pattern phenotype at a maximum density of four fish per litre. All tanks were filled with non-chlorinated water treated with AquaSafe™ (Tetra, VA, USA) and kept at 27 ± 1 °C under constant mechanical, biological, and chemical filtration. Room illumination was provided by ceiling-mounted fluorescent light tubes on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am). Three cohorts of zebrafish were used in the study to minimize the stress observed in subsequent experimental procedures. All animals used in this study were experimentally naïve and fed alcon BASIC™ Flakes (Alcon, Brazil) thrice daily. Animal experimentation in this study fully adhered to the National Institute of Health Guide for Care and Use of Laboratory and the protocols were approved by the Ethics Commission on Animal Use of the Federal University of Santa Maria under process number 106/2014.

2.2. Alarm substance preparation and exposure

Alarm substance was obtained from a conspecific skin extract as described elsewhere (Egan et al., 2009). Briefly, donor fish were anesthetized in water at 4 °C and quickly euthanized by decapitation. Alarm substance was extracted by damaging epidermal cells with 10–15 shallow slices on one side of the fish body with a razor blade. Cuts were carefully controlled to avoid drawing blood, which would contaminate the solution. The body was then washed in a Petri dish filled with 10 mL of distilled water and kept on ice. The dish was shaken gently to fully cover lacerated portions of the animals. The entire procedure was then repeated on the opposite side of the zebrafish and the aliquots were diluted to a 50% concentration. The exposure to alarm substance was performed using 3.5 mL/L for 5 min. Control group was handled in a similar manner, except that only distilled water was added. Both *WT* and *leo* were exposed to the skin extract obtained from their phenotypically similar donor fish as shown in Fig. 1A.

2.3. Behavioural experiments

All behavioural tests were recorded during the same time frame each day (between 10:00 am and 4:00 pm). The apparatuses were placed on a stable surface and the experimental procedures were performed with all environmental distractions kept to a minimum. The behavioural activities of fish were recorded for a single session

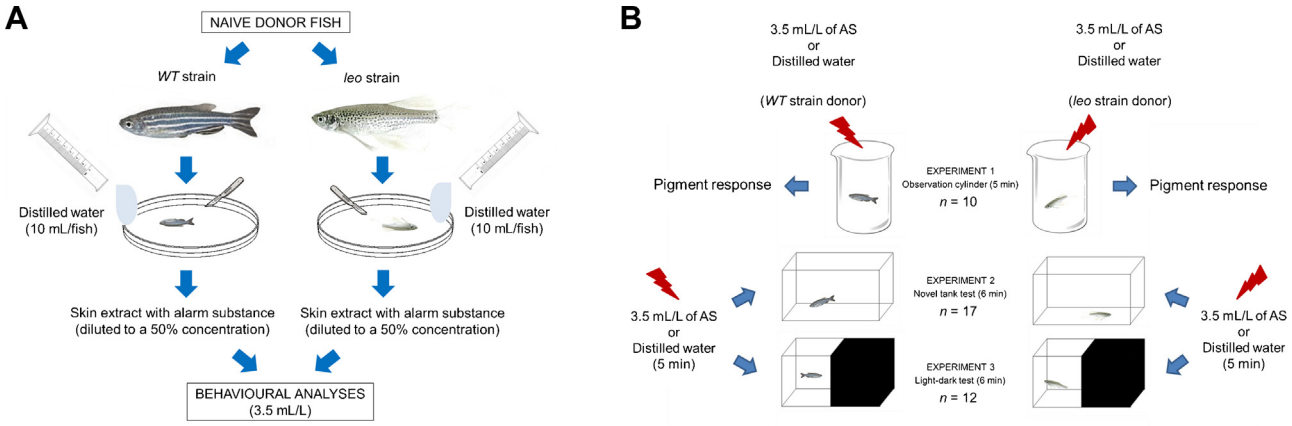

Fig. 1. Schematic representation of the experimental protocol. (A) The figure shows the method used for extracting the alarm substance of naïve donor fish from *WT* and *leo* strains. (B) Behavioural tests used for assessing the effects elicited by alarm substance in *WT* and *leo* (observation cylinder test—Experiment 1; novel tank test—Experiment 2; light–dark test—Experiment 3). Different cohorts were used for each experiment and animals tested in the observation cylinder were further used to analyse the influence of alarm substance on pigment response.

of 6 min, except for the observation cylinder test (Experiment 1), in which the behaviour was analysed during the exposure period (5 min) (Fig. 1B). The swimming location of zebrafish was tracked using a webcam connected to a laptop at a rate of 30 frames/s using appropriate video-tracking software (ANY-maze™, Stoelting CO, USA). After the experimental procedures, all fish were euthanized by as described above, except animals used for assessing the influence of alarm substance on body colouration. For measuring the colour intensity, we did not use any drug or ice-cold anaesthesia to avoid a possible interference on pigment response of zebrafish.

2.4. Experiment 1: observation cylinder test

The observation cylinder test (Experiment 1) was used to evaluate the vertical swimming activity during the exposure period based on the method previously reported (Cachat et al., 2013). A 500-mL glass cylinder (8 cm diameter, 10 cm height) was maximally filled with water and divided horizontally into two equal virtual portions (bottom and top). Due to the restrict dimensions of the observation cylinder, this test does not allow a complete investigation of the overall exploration of zebrafish. Nonetheless, it may represent an interesting methodological approach to reduce the amount of drugs that a single zebrafish may be exposed and to easily assess vertical drifts, burst swimming, and freezing, endpoints that are usually modulated by fear-inducing agents (Parra et al., 2009). Fish ($n = 10$ in each group) were placed individually in cylinders in the presence (experimental group) or absence (control group) of the skin extract and the following endpoints were measured: distance travelled (m), maximum speed (m/s), angular velocity (°/s), number and duration (s) of freezing bouts, number and duration (s) of erratic movements, number of entries and time spent (s) in bottom area, latency (s) to enter the top, number of entries and time spent (s) in top area. Freezing was defined as cessation of movement (except for gills and eyes) while fish presented increased opercular movements when immobile for at least 2 s. Erratic movements were defined as sharp changes in direction or velocity and repeated rapid darting behaviour. Freezing and erratic movements were manually quantified by two trained observers (inter-rater reliability >0.85). Both parameters are usually associated to increased fear/anxiety of zebrafish when aversive stimuli are present (Kalueff et al., 2013). Animals tested in the observation cylinder were further used to analyse the influence of alarm substance on body colouration.

2.5. Pigment response

The quantification of colour intensity of zebrafish was performed based on a protocol described previously (Cachat et al., 2013). After the exposure period, fish ($n = 10$ in each group) were quickly euthanized by decapitation and immediately placed on a white paper that served as a clear background. Photographs of each side of zebrafish body were taken with an 8.0-megapixel camera from a Samsung Galaxy S3™ smartphone mounted on a ring stand 8-cm distance above the fish to reduce potentially confounding variables (i.e., lighting, distance, subject size). The images were processed on a computer using the Image J 1.48 for Windows software to quantify the pigmentation. A region of interest representing a substantial part of zebrafish body with melanosome-filled area was selected over each fish image in a standardized manner at the lateral body surface. The mean grey value (MGV), ranging from 0 (black) to 255 (white), was then measured for this selected region. Results were normalized based on the pigment intensity of fish subtracted to the respective background and expressed as a saturation score index (SSI), calculated by the following formula: $SSI = \frac{1}{MGV} \times 100$.

2.6. Experiment 2: novel tank test

The novel tank test (Experiment 2) was used to analyse both locomotor and exploratory activity of zebrafish in a novel environment, which may reflect habituation to novelty stress (Rosemberg et al., 2011). Another cohort of zebrafish was exposed to alarm substance ($n = 17$ in each group) and further placed individually in a novel apparatus (25 cm length × 15 cm height × 6 cm width) divided into three horizontal portions (bottom, middle and top). The tank was filled with 2 L home tank water and the swimming behaviour was recorded. Locomotor activity was measured by distance travelled (m) and angular velocity (°/s). Fear/anxiety-related behaviours were determined by quantifying the number and duration (s) of freezing bouts as well as the number and duration (s) of erratic movements. Vertical exploration was assessed by the following endpoints: number of entries and time spent (s) in bottom area, latency (s) to enter the top, number of entries and time spent (s) in top area. The habituation profile was evaluated by assessing the number of entries and time spent (s) in top during the 6-min trial.

2.7. Experiment 3: light–dark test

The light–dark test was carried out based on the method described elsewhere (Maximino et al., 2010). The test apparatus (15 × 10 × 25 cm, height × depth × length) consisted in a glass tank divided into two equally sized dark and lit areas. Both compartments were delimited by opaque plastic self-adhesive films in black or white colours externally covering walls and floor. The apparatus was filled with 2 L home tank water and a third cohort of zebrafish was used for the behavioural analyses in the light–dark test. Fish ($n = 12$ in each group) were individually placed at the lit area. Fish were able to explore both compartments for 6 min and the following behaviours were determined: number of entries and time spent (s) in lit area, number and duration (s) of freezing bouts, latency (s) to enter the dark area and number of risk assessments. Risk assessment events were manually counted by two trained observers (inter-rater reliability >0.85) defined as a partial entry in the white compartment (i.e., the pectoral fin does not cross the midline) associated to a fast return to the dark compartment (Maximino et al., 2013; Kalueff et al., 2013).

2.8. Statistical analyses

Normality of data and homogeneity of the variances were analysed by Kolmogorov–Smirnov and Bartlett's tests, respectively. Data were expressed as mean ± standard error of the mean (S.E.M.) and analysed by two-way analysis of variance (ANOVA) using "strain" and "treatment" as factors. The transitions and time spent in top area across the trial (Experiment 2) were analysed by repeated measures ANOVA. The freezing data measured at Experiment 1 and Experiment 3 were expressed as median ± interquartile range and analysed by a nonparametric comparison of ranks using the Scheirer–Ray–Hare extension of Kruskal–Wallis test.

Principal component analysis (PCA) was performed to evaluate the potential correlation between variables from each behavioural task separately. The component matrix was further subjected to Varimax rotation with Kaiser normalisation. Components (or factors) with eigenvalue lower than 1 were disregarded, and measures with loadings greater than 0.3 were retained. Usually, the first principal component (PC1) explains the largest percentage of data variance. All other components (PC2, PC3, PCn) display decreasing amounts of the total variance. Data were run in SPSS 19 (IBM SPSS Statistics, version 19). Post hoc analyses were performed using

Table 1
Endpoints measured in *WT* and *leo* zebrafish subjected to the observation cylinder test. The body colour score was determined by optic density for control (Ctrl) and alarm substance (AS) groups.

Endpoint measured	Strain			
	WT		*Leo*	
	Ctrl	AS	Ctrl	AS
Number of erratic movements	2.70 ± 0.75^{a}	6.20 ± 1.34^{b}	2.60 ± 0.84^{a}	7.10 ± 1.99^{b}
Duration of erratic movements	1.29 ± 0.67^{a}	1.78 ± 0.38^{a}	0.73 ± 0.26^{a}	5.39 ± 1.43^{b}
Latency to enter the top area	18.99 ± 6.55^{a}	60.31 ± 19.91^{b}	79.76 ± 22.33^{b}	176.48 ± 19.70^{c}
Transitions to top area	$15.40 \pm 1.94^{a,b}$	$10.07 \pm 3.18^{a,b}$	21.70 ± 4.86^{a}	7.60 ± 2.04^{b}
Time in top area	$90.69 \pm 24.52^{a,b}$	148.72 ± 29.94^{a}	$64.02 \pm 17.61^{a,b}$	30.07 ± 9.95^{b}
Body colour score	1.21 ± 0.04^{a}	1.24 ± 0.03^{a}	0.96 ± 0.03^{b}	0.98 ± 0.03^{b}

Data were expressed as means ± S.E.M. and analysed by two-way ANOVA followed by Bonferroni's test as post hoc. Different letters indicate statistical differences for each endpoint at $p \leq 0.05$.

Bonferroni's multiple comparisons test when appropriate. The significance was set at $p \leq 0.05$.

3. Results

3.1. Observation cylinder test and pigment response

In the observation cylinder test (Experiment 1), no significant differences in distance travelled, maximum speed, angular velocity, number and duration of freezing were observed (data not shown). Table 1 shows the main results observed during the exposure period. The alarm substance significantly increased the number of erratic movements in *WT* and *leo* when compared to their respective control groups. While *leo* performed erratic movement for much longer, *WT* did not present significant changes for this parameter when alarm substance was present. The vertical swimming pattern analysis revealed that only *leo* exposed to alarm substance presented a significant decrease in the transitions to top area. Concerning the time spent in top area, no significant effects were detected for both strains exposed to alarm substance. Nevertheless, *leo* showed a significant decrease in time spent in top when compared to *WT* from alarm substance group. Both strains exposed to alarm substance displayed a significant increase in the latency to enter the top, but the values observed in *leo* were higher than those observed in *WT* groups. Photographs showing representative colouration of animals revealed that alarm substance did not significant change the pigment of both strains. However, we observed a higher saturation score index for *WT* when compared to *leo* groups (Table 1).

3.2. Novel tank diving test

In order to assess the locomotor and exploratory activities, another cohort of fish was further subjected to the novel tank test after the exposure period (Experiment 2). Although *WT* and *leo* did not present statistical differences in distance travelled, the alarm substance exposure significantly decreased the angular velocity in *WT* group. Moreover, the respective parameter was significantly higher in control *WT* when compared to both *leo* groups (Fig. 2A). Considering the number and duration of freezing bouts and erratic movements (Fig. 2B), both parameters were significantly increased after alarm substance exposure in *WT* but not in *leo*. Endpoint parameters of vertical exploration (Fig. 2C) showed that alarm substance induced a significant increase in the latency to enter the top only in *WT* and this value was higher than that obtained for both *leo* groups. *WT* exposed to alarm substance presented a significant decrease in the number of entries and time spent in top area. On the other hand, a significant increase in the time spent in top was observed for *leo* exposed to alarm substance. Compared to the *WT* controls, *leo* tended to show few transitions to upper area and spend less time in top. The habituation of both zebrafish strains to novelty was also evaluated during the 6-min trial (Supplementary Fig. 1). Temporal analyses revealed that the transitions to top presented a significant effect of strain ($F_{1,32} = 11.17$, $p = 0.0021$), time ($F_{11,352} = 4.301$, $p < 0.0001$) and interaction ($F_{11,352} = 3.600$, $p < 0.0001$), in which *WT* showed a faster transition to top and a higher number of entries in this area as compared to control *leo*. Furthermore, a significant effect of time ($F_{11,352} = 5.081$, $p < 0.0001$) was detected for the time in top during the test. In contrast to *leo*, *WT* displayed a robust increased in the time spent in upper area across the trial (Supplementary Fig. 1A). We further evaluated the effects of alarm substance on habituation profile of *WT* and *leo*. Basically, significant effects of time and treatment were observed for *WT* considering the number of entries ($F_{11,352} = 5.183$, $p < 0.0001$ and $F_{1,32} = 8.843$, $p = 0.0056$, respectively) and time spent in top area ($F_{11,352} = 5.942$, $p < 0.0001$ and $F_{1,32} = 7.181$, $p = 0.0115$, respectively). Although the transitions and time spent in top tended to increase across the trial for both groups, *WT* exposed to alarm substance presented few entries and spent less time in top during the test (Supplementary Fig. 1B). The analysis across the trial revealed significant effects of time ($F_{11,352} = 3.022$, $p = 0.0007$) and treatment ($F_{1,32} = 9.674$, $p = 0.0039$) for *leo* considering the time spent in top. Fish exposed to alarm substance spent more time in the upper area in comparison to control *leo* during the test. No significant differences were observed in the number of entries to top (Supplementary Fig. 1C).

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.beproc.2015.10.014.

3.3. Light–dark test

In the light–dark test (Experiment 3), significant differences between strains were verified. In comparison to control *leo*, untreated *WT* had more entries to the lit area of the apparatus as well as spent more time in the respective compartment. Exposure to alarm substance induced a significant decrease in both parameters for *WT*. A distinct effect was observed for *leo* after alarm substance exposure since an increased in the number of entries to lit area was detected, but the time spent this area did not significantly change (Fig. 3A). *WT* and *leo* presented a similar amount of freezing bouts. However, alarm substance induced a significant increase in the number of erratic movements in *WT* and *leo* when compared to their respective controls (Fig. 3B). When placed at a first glance in the lit compartment, untreated *leo* had a shorter latency to enter the dark area when compared to control *WT*. After exposure to alarm substance, *WT* presented a significant decrease in the latency to enter the dark area, while the values observed for *leo* remained unchanged. Additionally, after exposure to alarm substance, *leo* revealed a pronounced increase in the number of

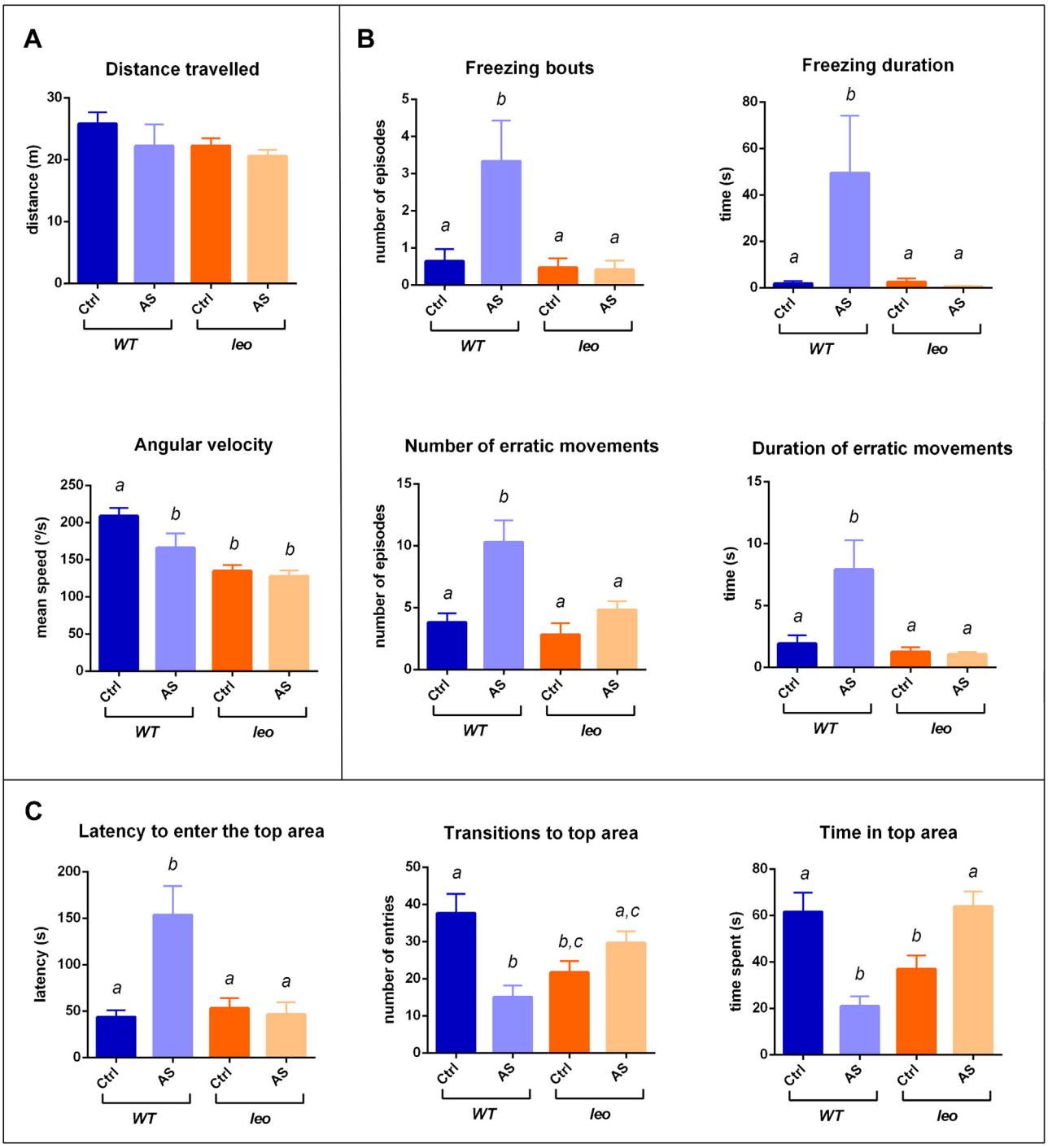

Fig. 2. Effects of alarm substance in *WT* and *leo* strains in locomotor activity and exploration in the novel tank test. (A) Locomotor activities. (B) Number and duration of freezing bouts and erratic movements. (C) V ertical exploration measured by the latency to enter the top, number of entries and time spent in top areas. Data are expressed as mean ± SEM and analysed by two-way ANOVA followed by Bonferroni's post hoc test. Distinct letters indicate statistical differences between experimental groups ($p \leq 0.05$. Ctrl = control; AS = alarm substance).

risk assessment episodes when compared to the respective control (Fig. 3C).

3.2. Principal component analysis (PCA) of behavioural endpoints

The analyses of animal behaviour require the compilation of a large set of variables in an integrated manner to allow a more precise interpretation. The use of PCA is a strategy that simplifies the structure of complex data sets. For the observation cylinder, novel tank and light–dark tests, the Kaiser-Meyer-Olkin measure of sampling adequacy were 0.565, 0.674, and 0.559, respectively. Bartlett's tests of sphericity were also significant for Experiment 1 ($\chi^2 = 362.283$, df = 66, $p < 0.0005$), Experiment 2 ($\chi^2 = 480.792$, df = 66, $p < 0.0005$) and Experiment 3 ($\chi^2 = 67.188$, df = 15, $p < 0.0005$), respectively.

For the observation cylinder test (Experiment 1), PCA extracted four principal components, which accounted for more than 79% of the total variance (Fig. 4). PC1 was associated with to burst swimming, with positive component loadings for distance travelled, angular velocity, number and duration of erratic movements

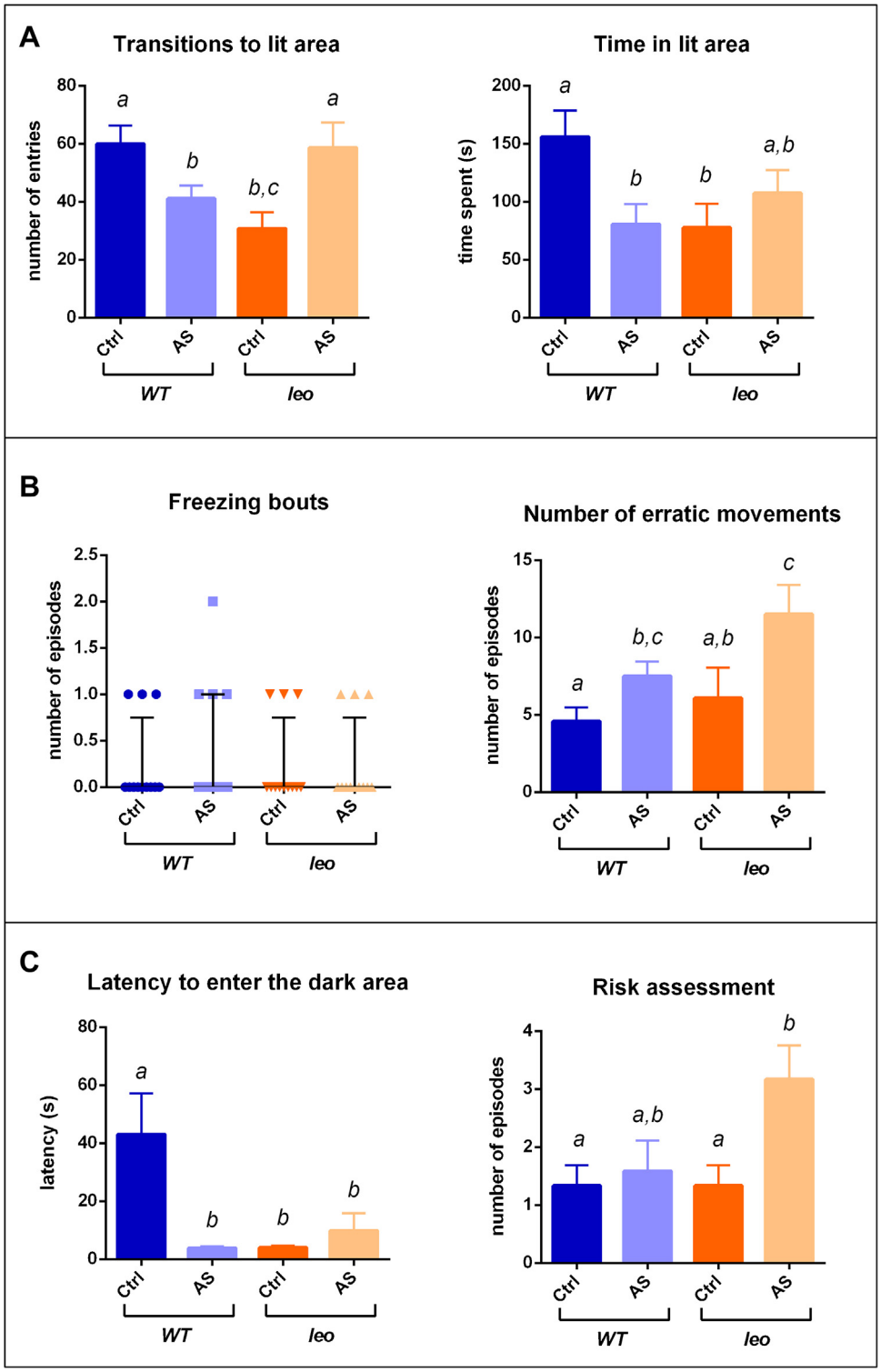

Fig. 3. Effects of alarm substance on behavioural pattern of *WT* and *leo* in the light–dark test. (A) Number of entries and time spent in lit area. (B) Number of freezing and erratic movement episodes. (C) Latency to enter the dark area and risk assessment. Data are expressed as mean ± SEM and analysed by two-way ANOVA, except the freezing data, which are analysed by a nonparametric comparison of ranks using the Scheirer–Ray–Hare extension of Kruskal–Wallis test ANOVA, followed by Bonferroni's post hoc test. Distinct letters indicate statistical differences between experimental groups ($p \leq 0.05$. Ctrl = control; AS = alarm substance).

and maximum speed. Two-way ANOVA yielded a significant effect of interaction ($F_{1,36} = 4.877$, $p = 0.0337$), treatment ($F_{1,36} = 18.46$, $p = 0.0001$), and strain ($F_{1,36} = 11.45$, $p = 0.0017$), with higher PC1 values for *leo* exposed to AS. PC2 was related to top swimming, being positively correlated with maximum speed, time in top area and transitions to top area, with negative component loadings for time in bottom area and latency to enter the top. A significant effect of strain was observed ($F_{1,36} = 16.50$, $p = 0.0003$), in which *leo* showed lower PC2 values in comparison to *WT* groups. PC3 was associated with vertical drifts, whereas PC4 was probably a representative component of fear-related behaviours. For PC3, two-way ANOVA revealed a significant effect of interaction ($F_{1,36} = 7.274$,

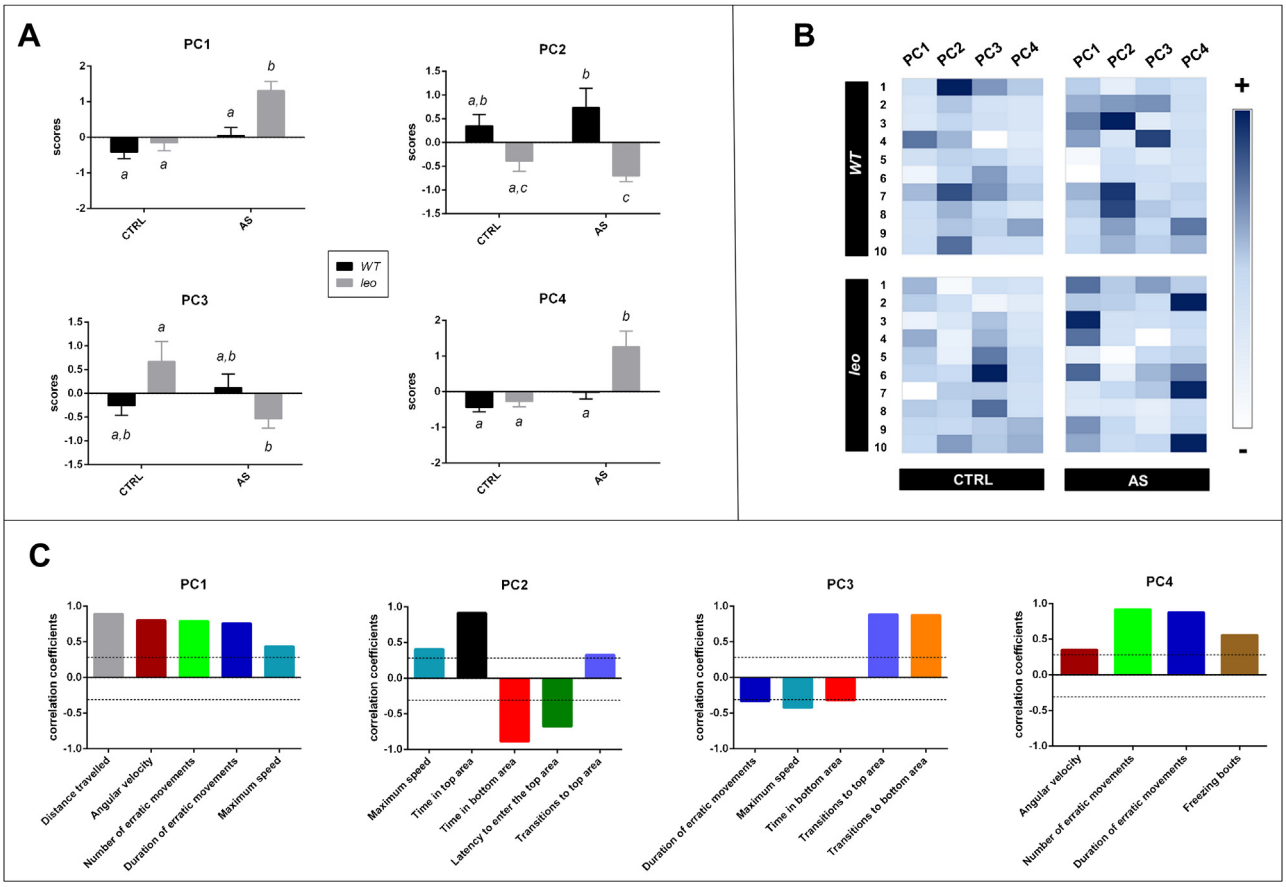

Fig. 4. Principal component analysis (PCA) for behavioural endpoints measured in the observation cylinder test. (A) Comparison of the values of four PC with eigenvalues greater than 1. Data are expressed as mean ± SEM and analysed by two-way ANOVA, followed by Bonferroni's post hoc test. Distinct letters indicate statistical differences between experimental groups ($p \leq 0.05$. Ctrl = control; AS = alarm substance). (B) Individual PC values for *WT* and *leo* zebrafish of each experimental group represented as a heat map diagram ($n = 10$). The intense blue colour indicates highest PC value, whereas the bright colours express lower PC values. (C) Correlation coefficients between the behavioural endpoints for each PC. Dashed lines represent cut-off points and only loadings greater than 0.3 or smaller than −0.3 are depicted.

$p = 0.0106$), while PC4 presented a significant effect of interaction ($F_{1,36} = 4.577$, $p = 0.0392$), treatment ($F_{1,36} = 14.34$, $p = 0.0006$), and strain ($F_{1,36} = 7.825$, $p = 0.0082$). Basically, *leo* exposed to AS showed decreased values for PC3 and higher PC4 values, respectively, when compared to its control.

For the novel tank test (Experiment 2), PCA extracted three principal components with eigenvalues higher than 1 that accounted for more than 67% of the total variance (Fig. 5). PC1 was associated with anxiety-like behaviours, with positive component loadings for time in bottom area, latency to enter the top, freezing duration, and maximum speed. It was negatively correlated with time in top area, transitions to top and bottom areas. Two-way ANOVA revealed a significant effect of interaction ($F_{1,64} = 27.36$, $p < 0.0001$) and both strains displayed contrasting PC1 values in the absence and in the presence of AS. PC2 was positively correlated with distance travelled, angular velocity, transitions to bottom area, and maximum speed being associated with locomotion. Although no statistical differences for PC2 values were detected, two-way ANOVA revealed a significant effect of interaction ($F_{1,64} = 6.396$, $p = 0.0139$), treatment ($F_{1,64} = 15.68$, $p = 0.0002$), and strain ($F_{1,64} = 17.81$, $p < 0.0001$) for PC3 values, in which *WT* exposed to AS had higher PC3 scores in comparison to the other groups. PC3 displayed a positive correlation with angular velocity, number and duration of erratic movements, and freezing bouts, suggesting a component of fear-related behaviours.

PCA extracted two principal components for the light–dark test (Experiment 3) (Fig. 6), in which PC1 was associated to locomotor/exploratory behaviours, with positive component loadings for transitions to lit area, number of erratic movements, risk assessment, and time in lit area being negatively correlated with freezing bouts. PC2 was associated to anxiolytic-like behaviours, showing a positive correlation with transitions to lit area, latency to enter the dark area, and time in lit area, with a negative component loading for risk assessment. These components accounted for more than 62% of the total variance of data.

For PC1, two-way ANOVA revealed a significant effect of interaction between treatment and strain ($F_{1,44} = 5.364$, $p = 0.0253$) with higher PC1 scores for *leo* exposed to AS when compared to the respective control group. Concerning PC2, significant effects of treatment ($F_{1,44} = 4.530$, $p = 0.0390$), strain ($F_{1,44} = 4.530$, $p = 0.0390$), and interaction ($F_{1,44} = 7.921$, $p = 0.0073$) were observed, in which untreated *WT* fish presented higher PC1 scores in comparison to the other groups.

The main results obtained for each endpoint measured considering their significance (p value) were summarized in Supplementary Fig. 2.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.beproc.2015.10.014.

4. Discussion

The comparison of the behavioural responses triggered by alarm substance is an interesting strategy to evaluate the relationship of a natural aversive agent with exploration and fear responses under different contexts (Russell, 1973). The influence of distinct

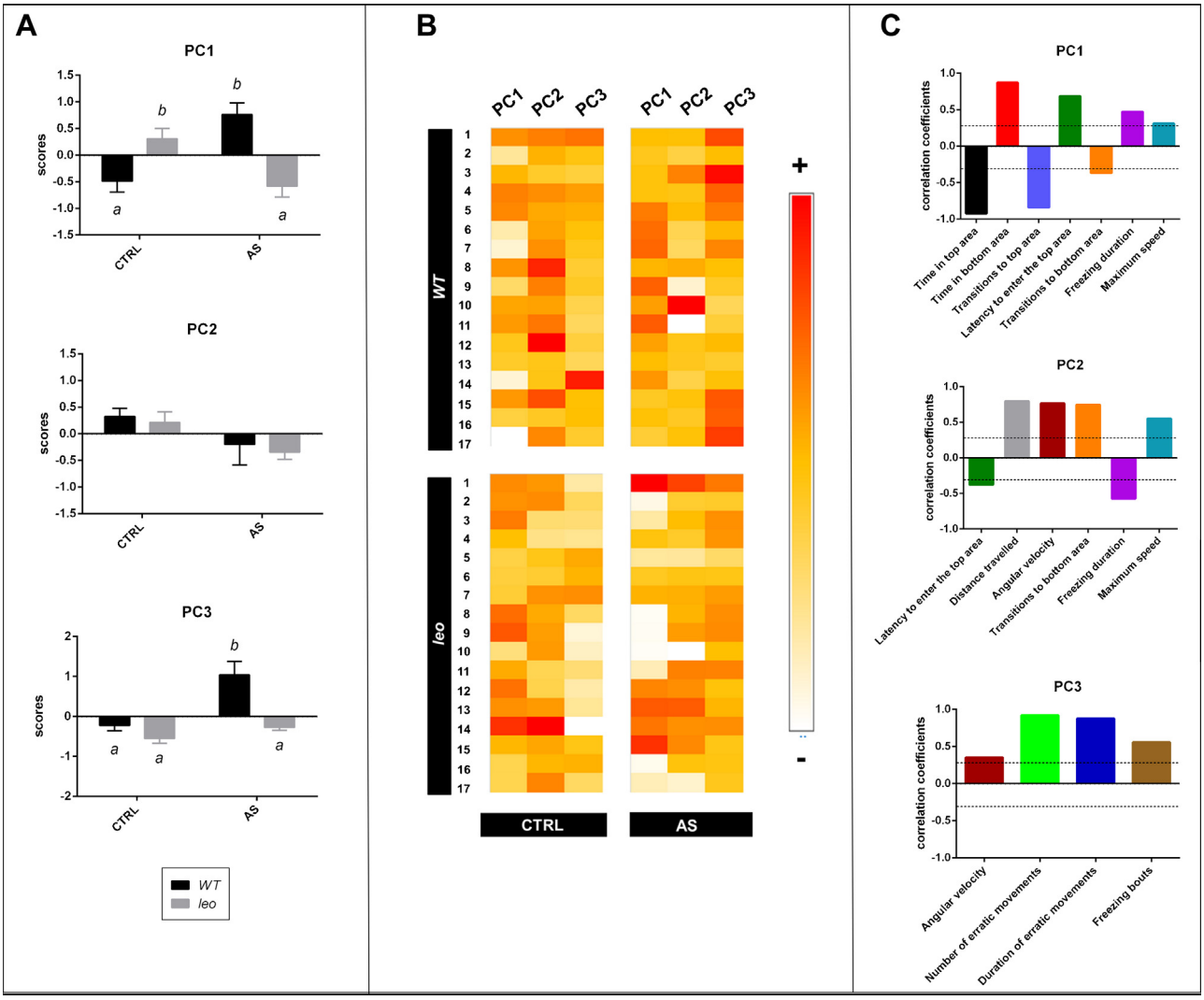

Fig. 5. Principal component analysis (PCA) for behavioural endpoints measured in the novel tank test. (A) Comparison of the values of three PC with eigenvalues greater than 1. Data are expressed as mean ± SEM and analysed by two-way ANOVA, followed by Bonferroni's post hoc test. Distinct letters indicate statistical differences between experimental groups ($p \leq 0.05$. Ctrl = control; AS = alarm substance). (B) Individual PC values for *WT* and *leo* zebrafish of each experimental group represented as a heat map diagram ($n = 17$). The intense red colour indicates highest PC value, whereas the bright colours express lower PC values. (C) Correlation coefficients between the behavioural endpoints for each PC. Dashed lines represent cut-off points and only loadings greater than 0.3 or smaller than −0.3 are depicted.

stimuli on fear responses depends on the nature, intensity and proximity of the threat and the severity of such behaviours may also be associated to differences in genetic background (Maximino et al., 2012). Five categories have been proposed to induce fear in animal models: predatory stimuli, mild electric shock, cues from conspecifics, novelty and intensive stimulus (Gray, 1987). It is known that Gray's 5 categories of fear-inducing stimuli are effective for modelling fear in zebrafish, but the influence of different contexts in *WT* and *leo* strains exposed to aversive chemical cues of conspecifics is unknown. In the present study, we analyse the behavioural profile of *WT* and *leo* acutely exposed to alarm substance using three experimental approaches: during the exposure period (Experiment 1—observation cylinder test); after exposure, in habituation to novel environments (Experiment 2—novel tank test); or after exposure, when placed into dark-bright contexts (Experiment 3—light–dark test). Adult zebrafish exposed to alarm substance display typical behavioural patterns. Acute exposure to skin extract of conspecifics can increase the frequency of erratic movements, freezing, bottom dwelling and scototaxis, which are associated to fear (Speedie and Gerlai, 2008; Parra et al., 2009). These behaviours are clearly adaptive and may serve as avoidance responses of stressful situations (Ahmed et al., 2011; Ferrari et al., 2010). During the exposure period, *leo* showed the most significant changes on swimming pattern with a decrease on vertical drifts and a higher frequency of erratic movements, suggesting increased defensive behaviours.When the animals were further tested in the novel tank or in the light–dark apparatus, a different behavioural pattern was verified. In comparison to their respective control groups, *WT* presented increased freezing and erratic movements associated to a decrease in vertical exploration, while *leo* spent more time in top area. Considering the light–dark test, both strains displayed increased erratic movements when compared to their controls, but *WT* had few transitions to the lit area and spent less time in the respective compartment. Thus, it is possible that different factors play a role in the acute responses of alarm substance in zebrafish.

The investigation of animal behaviour usually involves a large set of integrated variables that contribute to the phenotypes assessed (Loss et al., 2014). We have performed a PCA for each experimental design in order to simplify the structure of data set. Considering the increased values of PC1 and PC4 associated with the decreased values of PC3 observed in *leo* during alarm substance

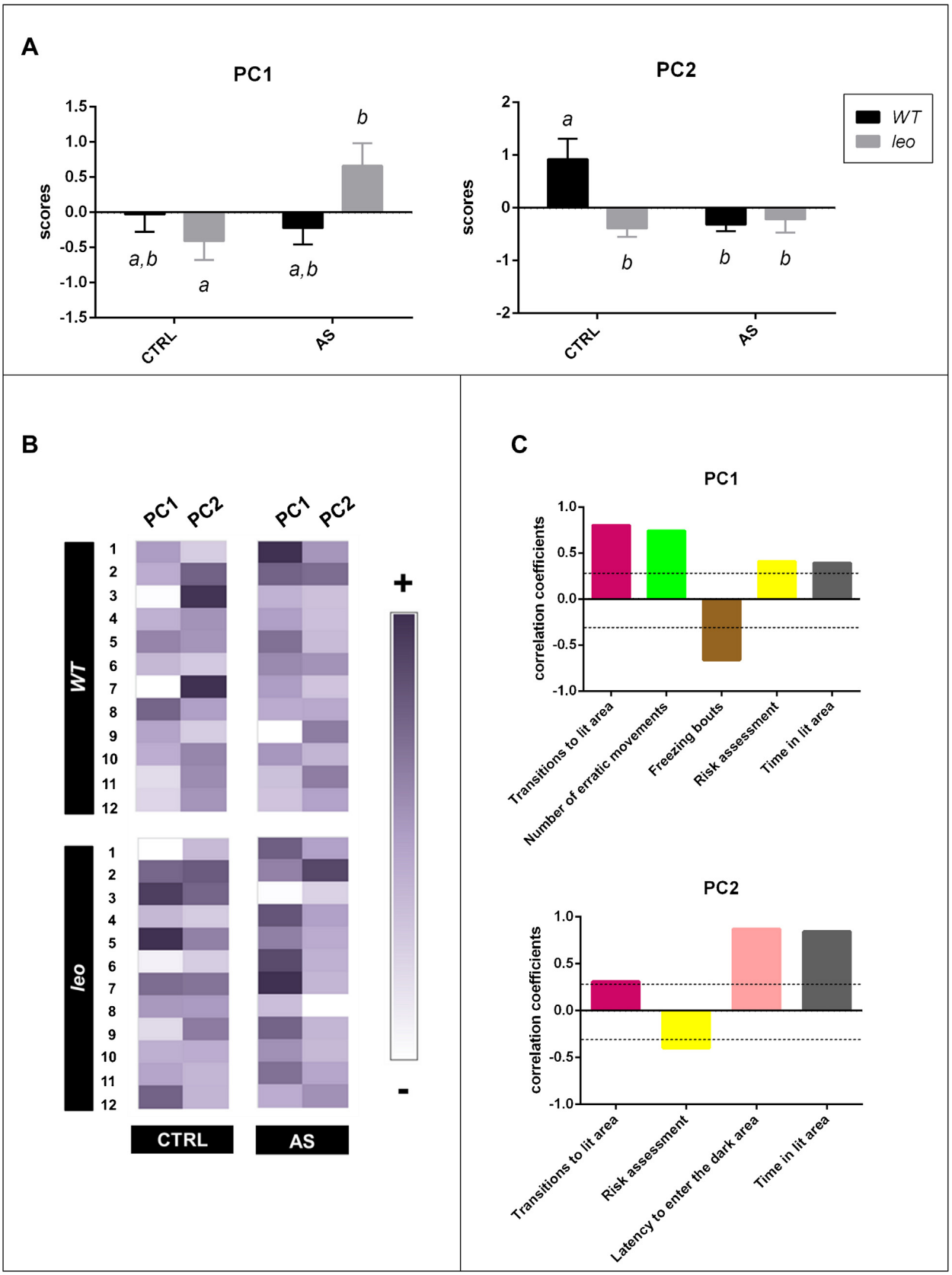

Fig. 6. Principal component analysis (PCA) for behavioural endpoints measured in the light–dark test. (A) Comparison of the values of two PC with eigenvalues greater than 1. Data are expressed as mean ± SEM and analysed by two-way ANOVA, followed by Bonferroni's post hoc test. Distinct letters indicate statistical differences between experimental groups ($p \leq 0.05$. Ctrl = control; AS = alarm substance). (B) Individual PC values for *WT* and *leo* zebrafish of each experimental group represented as a heat map diagram ($n = 12$). The intense purple colour indicates highest PC value, whereas the bright colours express lower PC values. (C) Correlation coefficients between the behavioural endpoints for each PC. Dashed lines represent cut-off points and only loadings greater than 0.3 or smaller than −0.3 are depicted.

exposure (Experiment 1), we suggest that the main responses observed are probably associated to increased burst swimming and fear-related behaviours with a concomitant reduction of vertical drifts. Three principal components were extracted for Experiment 2, which were associated to anxiety-like behaviours (PC1), locomotion (PC2), and fear-related behaviours (PC3). We do not have, at this moment, an exact explanation for the observed variation of angular velocity in *WT* and *leo* zebrafish exposed to alarm substance and further subjected to the novel tank test. However, this endpoint showed positive loadings in both PC2 and PC3 data. Angular velocity was positively loaded with distance, transitions to bottom area, and maximum speed (as shown by PC2 results). Moreover, it showed a positive correlation with number and duration of erratic movements, and freezing bouts (as verified by PC3 data). Probably, instead of a simple measure of locomotion, angular velocity may be associated with motor patterns of zebrafish influenced by different swimming activities (Rosemberg et al., 2012). It would be not surprising if different neurochemical mechanisms trigger such response, but future investigation to elucidate this point is needed. For the Experiment 3, PCA extracted two components, which may represent locomotion/exploration (PC1) and anxiety-like behaviours (PC2). In the light–dark test, we purpose an increase in locomotor/exploratory activities and a decrease in anxiolytic-like behaviours are associated to the acute effects of alarm substance in *leo* and *WT*, respectively. Therefore, these data represent the first evidence which demonstrates that the acute responses triggered by skin extract preparation are strain- and context-dependent in zebrafish.

Previous data demonstrated that both novelty stress and scototaxis are probably not associated to a same underlying construct (Blaser and Rosemberg, 2012; Rosemberg et al., 2011), a fact which is also corroborated with our findings. While in the novel tank test the motivational aspect is the surface escaping, the main stimulus in light–dark test is the avoidance to brightly lit areas (Blaser and Peñalosa, 2011). Another important difference is that the novel tank test evaluates vertical exploration during novelty stress which is susceptible to a gradual habituation response (Wong et al., 2010). The light–dark test measures a cryptic defensive pattern as a natural strategy for seeking protection and does not present intra- or inter-session habituation of white avoidance (Maximino et al., 2010). As previously reported (Chatterjee et al., 2014) we observe that both *WT* and *leo* have shown substantial differences in terms of exploration and anxiety, in which *leo* has decreased exploratory activity and increased anxiety-like behaviour. Assuming that the skin extract preparation induces fear (Ogawa et al., 2014) a plausible hypothesis is that *leo* should habituate less readily to novelty as well as display few transitions to lit area when compared to *WT*—predictions which are not supported by our results. Considering that the temporal analyses revealed a distinct vertical distribution, we postulate that alarm substance alters habituation to novel environments in a strain-dependent manner. In *WT*, impaired habituation could be associated to increased anxiety- and fear-related behaviours, while the enhanced habituation of *leo* could be related to a disruption of anxiety-like behaviour induced by alarm substance.A lthough these results are difficult to explain, studies from our group demonstrated that when *WT* is previously confined into a brightly lit environment (another model that induces fear) they habituate faster than fish confined into dark and transparent apparatuses in the novel tank (Rosemberg et al., 2011). These data reinforce the idea that the behavioural responses of zebrafish may not be easily predicted after an acute exposure to fear-inducing agents. Furthermore, since angular velocity was altered in novel tank test and that *leo* showed increased number of risk assessment in the light–dark task, we suggest that alarm substance can also modulate swimming pattern and exploration-related parameters.

The use of distinct strains serves as an attractive strategy to investigate the influence of genetic background on zebrafish behaviour. For example, a previous study using *Nadia*, *TM1* and *SH* strains demonstrated that relationships between activity level with tendency to approach a predator, tendency to move away from a shoal, and latency to feed after a disturbance is strain-dependent (Moretz et al., 2007). The *WT* and *leo* strains are both outbred populations separated by many generations, suggesting that they differ genetically at a wide range of loci. As example, *leo* strain presents mutations in the gene encoding connexins, proteins involved in intercellular communicationsin several tissues, such as brain, and associated to differences in the pigment pattern of *WT* and *leo* (Stehberg et al., 2012; Watanabe et al., 2006). Although we did not attempt to establish a specific relationship between genes and behaviour, our data reinforce the idea that genetic variations clearly contribute for complex behavioural patterns when different fear-inducing stimuli and contexts are involved.

Experimental manipulations that influence emotional state in zebrafish can darken or lighten their body colours by regulating aggregation or dispersion of melanosomes (Price et al., 2008). From a behavioural perspective, the pigment-based colour patterns of fish can be an indicator of their role as a potential mate or competitor, or reveal their escape ability in the eyes of predators. Several factors can influence the level of pigmentation, such as age, seasonally, and even genetics. Since the colour intensity of pigment-containing cells is controlled by both the nervous and endocrine systems, changes on the colour intensity may predict motivational state (assuming honest signalling) (Price et al., 2008). We demonstrate that the colour intensity was more pronounced in *WT* group but no differences in comparison to control were observed in animals exposed to alarm substance from both strains. These results suggest that the pigments are more widespread presenting a less intense colour in *leo*. Although the stimuli triggered by chemical cues from conspecifics are able to modulate defensive behaviours, they do not influence pigment response in zebrafish.

5. Conclusion

Overall, we demonstrate that the behavioural responses of alarm substance in zebrafish are strain- and context-dependent. These data may be relevant for assessing the motivational aspects that drive natural behaviours of zebrafish (e.g., diving response, habituation to novelty, bright avoidance) under aversive situations. Considering that *WT* and *leo* present robust differences in terms of exploration and anxiety, the selection of strain used may represent a refinement for examining the effects of several experimental manipulations on defensive behaviours. However, we emphasize that the studies of the neural basis associated to the responses triggered by chemical cues from conspecifics, as well as the potential modulation of specific genes, are still a challenge. Our data also provide implications for future studies aiming to characterise the chemical properties of alarm substance of *WT* and *leo*, as well as the susceptibility of each strain to chemical cues and the putative relevance in terms of natural behaviours. Considering that many factors are involved in individual response to fear-inducing agents, we point the necessity to investigate which stimuli are controlling the endpoints measured and which brain area(s) is (are) involved in the behavioural phenotypes observed.

Conflict of interest

The authors have declared that no competing interests exist.

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5. CONCLUSÕES

Em suma, a presente Dissertação demonstra que as respostas comportamentais induzidas pela substância de alarme em peixe zebra são dependentes de linhagem e contexto. Essa conclusão pode ser sustentada pelos seguintes dados:

- Durante o período de exposição, ambas as linhagens apresentaram um aumento do número de movimentos erráticos, mas somente a linhagem *leo* diminuiu a frequência de transições verticais.
- O padrão de resposta pigmentar não é alterado pela substância de alarme em ambas as linhagens;
- No teste do tanque novo, a substância de alarme não alterou a locomoção, mas promoveu um aumento significativo na frequência de comportamentos defensivos em *WT* e um maior tempo de exploração vertical em *leo*;
- No teste claro-escuro, a substância de alarme promoveu um aumento no número de movimentos erráticos em ambas as linhagens, uma menor exploração a ambientes claros em *WT* e um maior número de episódios de avaliação de risco em *leo*;
- As principais respostas associadas aos efeitos da substância de alarme para os três testes foram: nado acelerado (teste do cilindro de observação), comportamento tipo ansiedade (teste do tanque novo) e locomoção/exploração (teste do aquário claro-escuro).

6. PERSPECTIVAS DO ESTUDO

Este trabalho identificou que os efeitos comportamentais promovidos pela exposição aguda à substância de alarme são dependentes do contexto e da linhagem de peixe zebra estudada. Dessa maneira, utilizando as linhagens *WT* e *leo*, as perspectivas do estudo são:

- Avaliar os efeitos da exposição crônica à substância de alarme sobre a atividade locomotora, exploratória e no comportamento tipo ansiedade;
- Explorar as ações promovidas pela exposição aguda e crônica à substância de alarme no comportamento agonístico;
- Avaliar a influência da substância de alarme no comportamento de grupo e interação social;
- Verificar se a modulação de parâmetros relacionados ao estresse oxidativo cerebral contribui para os mecanismos de ação de resposta promovida pela substância de alarme (aguda e crônica);
- Analisar a influência da substância de alarme sobre parâmetros da neurotransmissão colinérgica e purinérgica, as quais estão relacionadas com comportamentos de medo e ansiedade em vertebrados;
- Investigar o efeito da exposição à substância de alarme sobre parâmetros relacionados ao estresse, tais como níveis de cortisol total e expressão gênica do fator liberador de corticotropina e do receptor de glicocorticoide em SNC;
- Padronizar e validar no laboratório um modelo com alto valor fenomenológico (de face) para estudos relacionados ao transtorno de estresse pós-traumático utilizando a substância de alarme como estímulo aversivo para o condicionamento de medo.

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ANEXO A – CARTA DE APROVAÇÃO DA CEUA

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM

CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Mecanismos de ação da substância de alarme em diferentes linhagens de peixe-zebra (Danio rerio): Uma análise comportamental e bioquímica."

Número do Parecer: 106/2014

Pesquisador Responsável: Prof. Dr. Denis Broock Rosemberg

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

OBS: Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

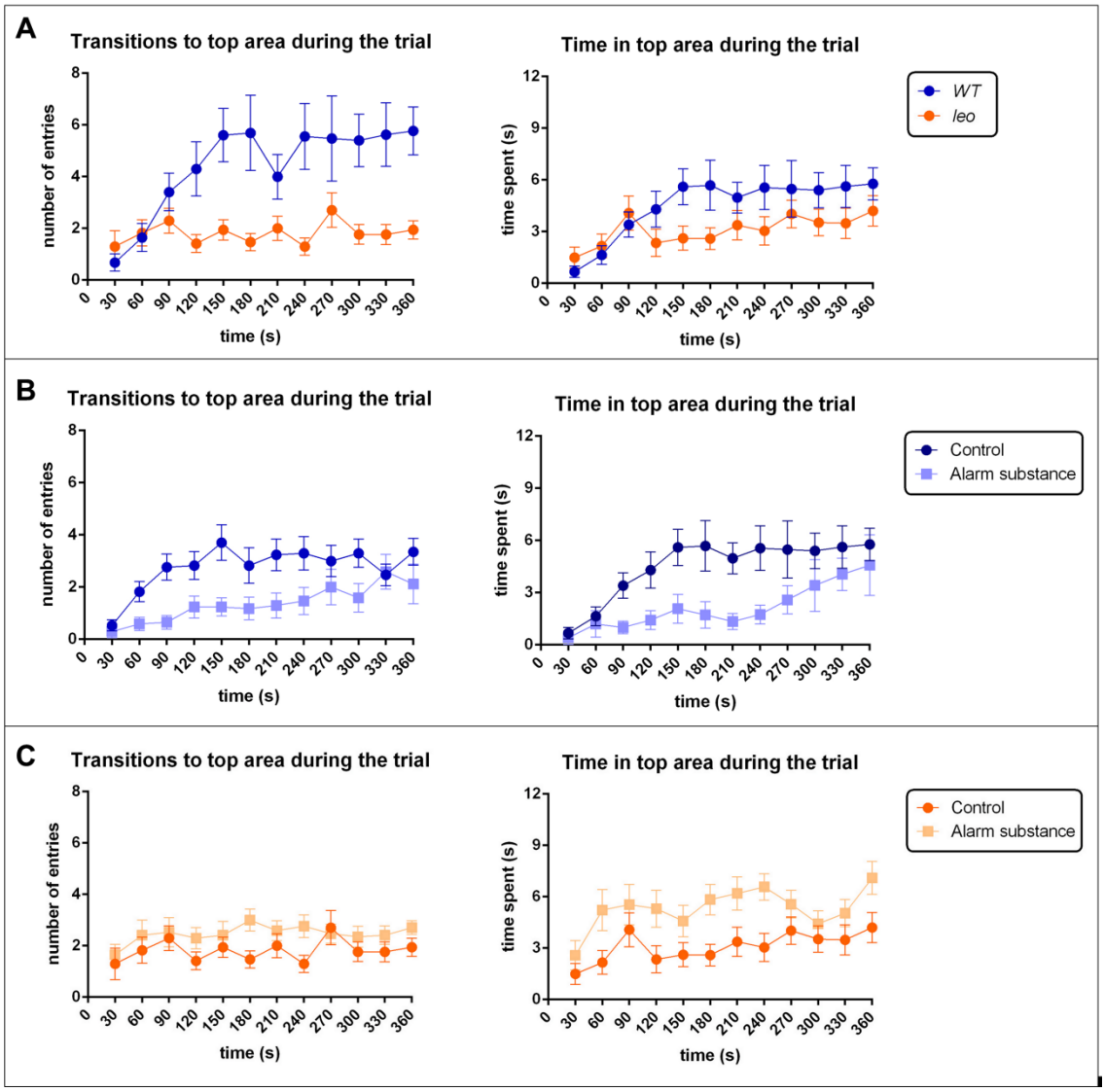
DATA DE APROVAÇÃO: 01/10/2014.

Santa Maria, 01 de outubro de 2014.

Vania Lucia Loro

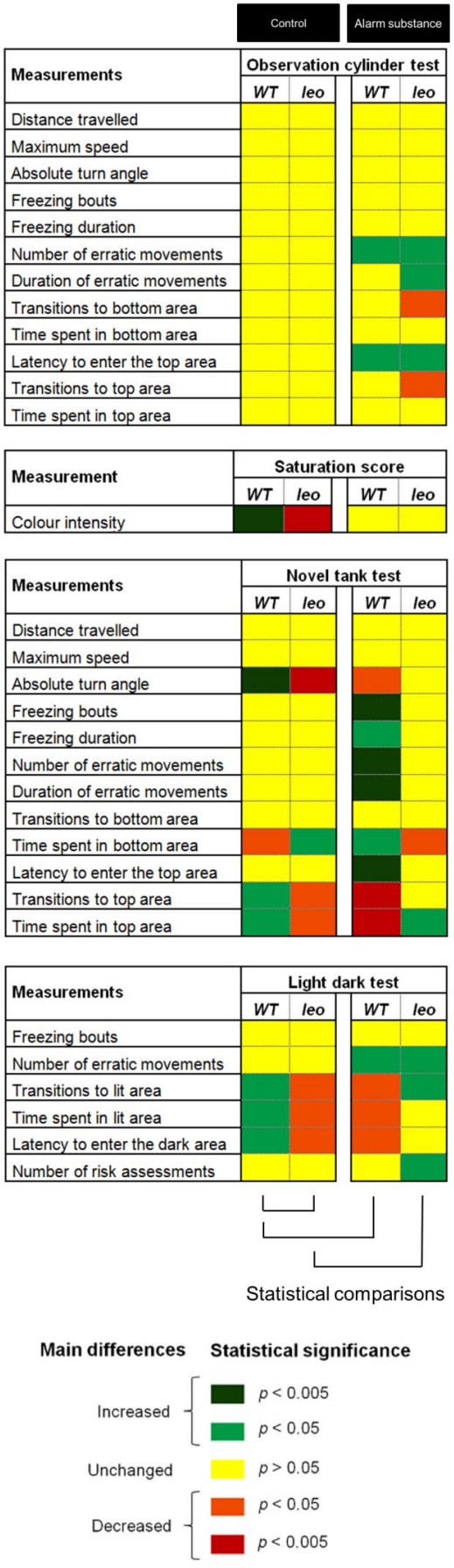
Prof.ª Dr.ª Vania Lucia Loro
Vice-Coordenadora da Comissão de Ética no Uso de Animais- UFSM

ANEXO B – FIGURA SUPLEMENTAR 1



Supplementary Figure 1: Alarm substance affects habituation to novelty in both *WT* and *leo* strains. (A) Comparison of the distinct habituation profile of untreated *WT* and *leo* assessed by the number of entries and time spent in top area during the 6-min trial. (B) Effects of alarm substance in the habituation profile of *WT*. (C) Effects of alarm substance in the habituation profile of *leo*. Data are expressed as mean ± SEM and analysed by repeated measures ANOVA, followed by Bonferroni's post hoc test considering $p \leq 0.05$ as significant.

ANEXO C – FIGURA SUPLEMENTAR 2



Supplementary Figure 2: Representative colour plots summarizing the endpoints measured in the observation cylinder test, pigment response, novel tank test and light-dark test. The figure shows the main differences between control *WT* and *leo* as well as the effects elicited by alarm substance exposure in *WT* and *leo* when compared to their respective control groups. The results were represented by distinct colours, which describe the overall results of the experiments and the statistical significance. Deceased values are shown as orange ($p < 0.05$) and red ($p < 0.005$) boxes, while the light green ($p < 0.05$) and dark green ($p < 0.005$) colours indicate increased parameters. When the behavioural parameters measured did not significantly differ ($p > 0.05$) they were represented as yellow boxes.