Sensory ecology of Ostariophysan alarm substances

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Abstract

Chemical communication of predation risk has evolved multiple times in fish species, with the conspecific alarm substance (CAS) contemporaneously being the most well understood mechanism. CAS is released after epithelial damage, usually when prey fish is captured by a predator, and elicits neurobehavioral adjustments in conspecifics which increase the probability of avoiding predation. As such, CAS is a partial predator stimulus, eliciting risk assessment-like and avoidance behaviors, and disrupting the predator sequence. The present paper reviews the distribution and putative composition of CAS in fish, and presents a model for the neural processing of these structures by the olfactory and the brain aversive systems. Applications of CAS in the behavioral neurosciences and neuropharmacology are also presented, exploiting the potential of model fish (e.g., zebrafish, guppies, minnows) on neurobehavioral research.

Keywords: Alarm substance; Alarm signals; Disturbance signals; Fish; Ostariophysi

1. Introduction

Each species possess specific (tactile, acoustic, chemical) and cross-model communication channels for different finalities. For fish, chemical communication in the aquatic environment causes behavioral adjustments and shifts that require the transfer of adaptive information between senders and receivers. This communication has different aims: demarcation of territory, sexual attraction, food signaling, danger signals, among others (Liley, 1982).

Alerting conspecifics to the presence of predators increases their chances of surviving an attack by avoiding an encounter or escaping capture. The methods and resources available to alert conspecifics are species-specific and vary according to context and other factors. In order for these "predator alert" signals to be conveyed, the central nervous system and sense organs evolved (Pfeiffer, 1977; Liley, 1982). In fish, chemical communication of predation risk has evolved multiple times in the form of conspecific alarm substances (CASs) and disturbance signals (DSs; see Box 1).

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Box 1. Disturbance signals in fish

Behavioral responses of fish depend on the type of threatening chemical cues. Fish use a combination of information and the context of the situation to determine their defensive strategies. While CASs are signals that are released after (potentially terminal) capture, *disturbance signals* (DS) involves the communication of predator threat without damage to the animal; one example of a disturbance signal is the alarm call of many birds and macaques, which, after visually detecting a predator, acoustically communicate this to their mates.

Chemical DSs have been demonstrated in some fish species. Unstressed Nile tilapia (*Oreochromis niloticus*) and jundiá (*Rhamdia quelen*) exposed to water in which a conspecific received handling stress show increased cortisol levels (Barcellos et al., 2011). Perhaps more ecologically relevant is the observation that information regarding predators is also transferred. In the pacu *Piaractus mesopotamicus*, the sight of a predator elicits antipredator behavior; unstressed pacus exposed to water in which a conspecific was visually exposed to a predator avoid the chemical stimulus (Jordão & Volpato, 2000). Zebrafish show increased whole-body cortisol after being exposed to water from a conspecific that had visual contact with a predator fish (Oliveira et al., 2013); interestingly, even when whole-body cortisol is *not* increased in animals with visual contact with the predator, these endocrine responses are still observed in animals receiving chemical cues from these “donor” animals” (Barcellos et al., 2014). These results suggest the existence of chemical cues that are released in the water by non-injured fish (disturbance signals) to warn conspecifics of the presence of predators. Another interesting finding is that zebrafish visually exposed to a predator display antipredator behaviors, including tighter shoaling, that in its turn trigger defensive behavior in conspecifics which did not originally see the predator (Oliveira et al., 2017). The relationship between cortisol and behavioral responses, however, has not yet been determined, opening an interesting avenue of investigation.

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Conspecific alarm substances were first described by Karl von Frisch (1938, 1941) using the Eurasian minnow *Phoxinus phoxinus*. von Frisch described that these fish "seem terrified" when a skin extract produced from conspecifics was introduced, fleeing a short distance "in confusion, increasing shoal cohesion, and retreating. Later, it was observed that most Ostariophysans display a "fright-like" alarm response towards CAS (von Frisch, 1941; Pfeiffer, 1977; Smith, 1992; Jesuthasan & Mathuru, 2008; Døving & Lastein, 2009), although considerable interspecific variation is observed (see Box 2 for experimental findings on zebrafish and fathead minnows): some species can display thrashing against the tank bottom, swimming with their heads against the bottom and their bodies at an angle to the floor; some species may display prominent freezing (i.e., become motionless for extended periods of time); some species may display extensive bottom-dwelling, with special gases in which the fish starts spitting gas for a considerable time; and some may display surfacing behavior, crowding together at the surface and attempting to jump out of the water (Pfeiffer, 1977). Later, it has also been shown that some non-Ostariophysan fish also display CAS.

Box 2. What are the behavioral effects of CAS?

Fear can be defined as an adaptive reaction to an aversive stimulus that is necessary for moments of distal or proximal threat (such as the presence of a predator), generating either defensive fighting, freezing, or fleeing (Fanselow & Lester, 1988; Perusini & Fanselow, 2015). While exposure to certain threat is expected to induce fear-like behaviors, partial predator stimuli such as odors tend to elicit more risk assessment, but may also produce strong species-specific reaction defenses, including freezing and avoidance (Dielenberg & McGregor, 2001). Since CAS is released by wounded fish, and not by the predator, it should be considered a partial predator stimulus, and therefore it is not clear whether the behavioral profile induced by CAS is fear-like.

While CAS behavioral effects have been described in many Ostariophysan and non-Ostariophysan species, as described above, a more careful observation of the behavioral effects in laboratory setting has been made using zebrafish and fathead minnows. In the first species, CAS

has been shown to elicit erratic swimming, freezing, shoal aggregation, jumps, and bottom-dwelling (Table 1). Results from the literature are inconsistent, and appear to be associated with whether the substance is present or absent during testing (i.e., whether animals are observed during or after CAS exposure), and whether animals are tested alone or in groups. When animals are tested alone, there is a consistent increase in bottom-dwelling, freezing, and erratic swimming during exposure (Table 1), while there is considerable variation in those endpoints after exposure (Table 1). It is not clear what procedural variations lead to which effects *after* exposure, but certainly there is great variation in the literature. Zebrafish is a shoaling species, and, in nature, CAS is expected to function in shoals. As such, increased shoaling responses are consistently observed during CAS exposure (Table 1; Speedie & Gerlai, 2008; Lima et al., 2016; Canzian et al., 2017; Choi et al., 2017).

i1. Fish tested alone

Endpoint	**During exposure**	**After exposure**
Geotaxis (Bottom-dwelling)	Zebrafish	Zebrafish
	↑ (Nathan et al., 2015a; Eachus et al., 2017)	↑ (Schirmer et al., 2013; Quadros et al., 2016)
	Fathead minnow	0 (Nathan et al., 2015a)
	Not tested	
		Fathead minnow
		Not tested
Freezing	Zebrafish	Zebrafish
	↑ (Ogawa et al., 2014; Nathan et al., 2015a; Maximino et al., 2018)	↑ (Egan et al., 2009; Quadros et al., 2016)
		0 (Nathan et al., 2015a)
	Fathead minnow	
	↑ (Lawrence & Smith, 1989)	Fathead minnow
		Not tested

Erratic swimming	Zebrafish ↑(Ogawa et al., 2014; Nathan et al., 2015a; Maximino et al., 2018) Fathead minnow ↑ (Lawrence & Smith, 1989)	Zebrafish 0 (Nathan et al., 2015a) ↑ (Egan et al., 2009) Fathead minnow Not tested
Scototaxis (Dark preference)	Not tested	Zebrafish ↑ (Maximino et al., 2014; Lima et al., 2016; Quadros et al., 2016) 0 (Mansur et al., 2014) Fathead minnow Not tested
Risk assessment (light/dark test)	Not tested	0 (Maximino et al., 2014; Quadros et al., 2016) Fathead minnow Not tested
2. Fish tested in shoals		
Endpoint	**During exposure**	**After exposure**
Geotaxis	Zebrafish 0 (Speedie & Gerlai, 2008) ↑ (Canzian et al., 2017; Ruhl et al., 2017) Fathead minnows ↑ (Yunker et al., 1999)	Not tested
Freezing	Zebrafish 0 (Speedie & Gerlai, 2008; Canzian et al., 2017) Fathead minnows	Not tested

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	Not tested	
Erratic swimming	Zebrafish	Not tested
	↑ (Speedie & Gerlai, 2008; Canzian et al., 2017)	
	Fathead minnows	
	Not tested	
Shoaling	Zebrafish	Not tested
	↑ (Speedie & Gerlai, 2008; Lima et al., 2016; Canzian et al., 2017; Choi et al., 2017)	
	Fathead minnows	
	Not tested	

In addition to these effects, other important behavioral adjustments were also observed, in zebrafish, during or after CAS exposure. These adjustments include fear-induced analgesia (Maximino, 2011; Maximino et al., 2014), an inhibition of nocifensive behavior that is thought to allow receivers to flee even when they are injured. Moreover, CAS also serves as a platform for learning, as animals learn to avoid areas or cues which were previously associated with *shreckstoff* exposure (Hall & Suboski, 1995a, 1995b; Ruhl et al., 2017; Maximino et al., 2018). Finally, as appears to be the case with predator odors in rodents (Blanchard et al., 2003), CAS can also produce long-term (time-dependent) sensitization of defensive responses (Lima et al., 2015, 2016), suggesting a basis for the creation of models for PTSD (Section 9).

In Ostariophysans, CAS is produced on specialized club cells in the epidermis (Pfeiffer, 1977) that, when damaged, release the substance (a “signal”, in Smith’s (1992) terminology) into the water, initiating alarm reactions in conspecifics. Whether or not the animal that produces and releases CAS (termed “sender”, follow Smith (1992)) also benefits from the transmission is as of yet

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unresolved. von Frisch (1941) was also the first to demonstrate that CAS is an olfactory signal, demonstrating the importance of this sensory channel for ecological interactions in Ostariophysans.

While the term "alarm substance" is now widespread, in order for a given mechanism to classify as an alarm signal, it needs to be produced by the sender when it detects threat; it also needs to be detected by receivers (ideally conspecifics) in a way that they react in a way that is similar to their reaction to actual threats (Smith, 1992). Indeed, CAS is best defined as a "partial predator stimulus" (Dielenberg & McGregor, 2006) in that it does not faithfully signals the presence of a predatory threat, but instead signals a potential threat. As such, differently from other ecologically relevant sensory signals, CAS *increases* uncertainty instead of reducing it, producing behavioral adjustments that decrease the probability of an (uncertain) capture.

The present review attempts to capture the complexity of responses to conspecific alarm substances in fish, including the behavioral characteristics of the alarm reaction, the mechanisms of its detection by the olfactory system, the mechanisms of response production by the aversive brain system, and potential applications in the fields of behavioral neuroscience and neuropsychopharmacology.

2. Phylogenetic distribution of CAS

First described in cyprinids (von Frisch, 1938, 1941), later it was shown that CAS is produced by most fish from the superorder Ostariophysi (Pfeiffer, 1977). The presence of club cells, a specialized epidermal cell with high cytoplasm-to-nucleus ratio that lacks porous openings to the exterior, is thought to be crucial in this superorder (Pfeiffer, 1977). Damage to these club cells – normally after being captured by a predator – leads to the release of alarm substance; as such, CAS cannot be released voluntarily.

Alarm reactions have also been observed in a few non-Ostariopysan fishes, although that appears to be an exception (Pfeiffer, 1977). While most percomorpha did not display an alarm reaction

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or club cells (Pfeiffer, 1977), alarm reactions have been described in the perciforms Nile tilapia (*Oreochromis niloticus*) (Sanches et al., 2015; Silva et al., 2015), Mozambique tilapia (*Oreochromis mossambicus*) (Jaiswal & Waghray, 1990), convict cichlids (*Amatitlania nigrofasciatus*) (Alemadi & Wisenden, 2002), and in the fillfrin goby (*Bathygobius soporator*) (Barreto et al., 2014), suggesting that this response evolved independently multiple times in this clade.

Curiously, some poeciliids display an alarm reaction to conspecific skin extracts, even if club cells cannot be identified in the species (Pfeiffer, 1977). Similarly, the medaka (*Oryzias latipes*, Beloniformes) show freezing episodes to conspecific skin extract and increased whole-body cortisol levels, but appear to lack club cells (Mathuru, 2016). However, since there are no positive markers to club cells in histological slides, it is hard to properly ascertain the lack of such cells. Nonetheless, it seems that alarm substances evolved multiple times in fish, and was probably present at the root of the Ostariophysan superorder. Most alarm substances appear to be released by damage to club cells, but the existence of alarm reactions without club cells is also possible, especially in non-Ostariophysi. Moreover, methodologically it is difficult to separate club cell contents from traces of blood, and blood itself is able to induce a behavioral response, in tilapia, that is similar to the alarm reaction (Barreto et al., 2013). Therefore, an explanation of the alarm reaction observed in medaka and poeciliids is the presence of blood traces in the extract. These results also underline the need for parsimony in interpreting results obtained from CAS obtained from skin extracts: while it is unlikely that blood is the only component of these reactions, because components which are found in CAS also elicit alarm reactions (see below), the possibility that blood traces are responsible for the behavior should be kept in mind.

3. Chemical composition of conspecific alarm substances

The exact composition of CAS is as of yet unknown. Hüttel (1941) already reported that nitrogen compounds were likely to be important, assuming that purine- and pterin-like substances to

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be the main components of CAS. Attempts to describe a single compound all failed, and soon it was clear that multiple odorants make up conspecific alarm substances (Døving & Lastein, 2009). Alarm reactions could be elicited in zebrafish (*Danio rerio*), fathead minnows (*Pimephales promelas*), and finescale dace (*Chrosomus neogaeus*) when exposed to hypoxanthine-3-*N*-oxide (H3NO) or the functionally similar pyridine-*N*-oxide, but not to structurally similar molecules that lacked nitrogen oxide-based functional groups (Pfeiffer & Riegelbauer, 1984; Brown et al., 2000; Parra et al., 2009; Mathuru et al., 2012). Nonetheless, at least in zebrafish H3NO elicits some, but not all, components of the alarm reaction (Parra et al., 2009; Mathuru et al., 2012), and therefore it is expected to be one in a cocktail of substances in CAS.

Further clues were obtained by fractioning the brute skin extract of zebrafish by anion exchange chromatography and high-performance gel filtration, which produced fractions which were able to elicit clear behavioral responses in zebrafish (Mathuru et al., 2012). A fraction with high molecular weight was found, through mass spectrometry, to contain long polymers, including chondroitin glycosaminoglycans (Mathuru et al., 2012). Indeed, heavy molecular weight chondroitin sulfate fragments were able to induce a robust alarm reaction in zebrafish (Mathuru et al., 2012), as well as increasing *fos* expression in the dorsomedial olfactory bulb (DeCarvalho et al., 2013). These results suggest that both glycosaminoglycans and nitrogen oxide-based purines and pterins to be important for CAS signaling.

4. CAS disrupts the predation sequence

Irrespective of what is the evolutionary pressures which resulted in the evolution of an alarm signal in fish, it has long been understood that CAS disrupts the predation sequence at multiple points (von Frisch, 1941; Smith, 1992). Lima & Dill (1990) suggested that a predation sequence develops from a predator-prey encounter that (if the prey is first detected by the predator) can evolve to an attack that is followed by either escape or capture (Figure 1). Using Fanselow's terminology

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(Fanselow & Lester, 1988; Perusini & Fanselow, 2015), from pre-encounter defensive behaviors (meal reorganization, careful exploration of non-safe environments) the sequence develops to post-encounter defense (aimed at avoiding detection and attack), followed by circa-strike defensive behavior (including attempts to flee or attack the predator)(Figure 1). Smith (1992), in a now classical review, suggested that CAS acts at the second level (post-encounter defense) to decrease the probability that the conspecific is detected first and increase the probability that it detects the predator first.

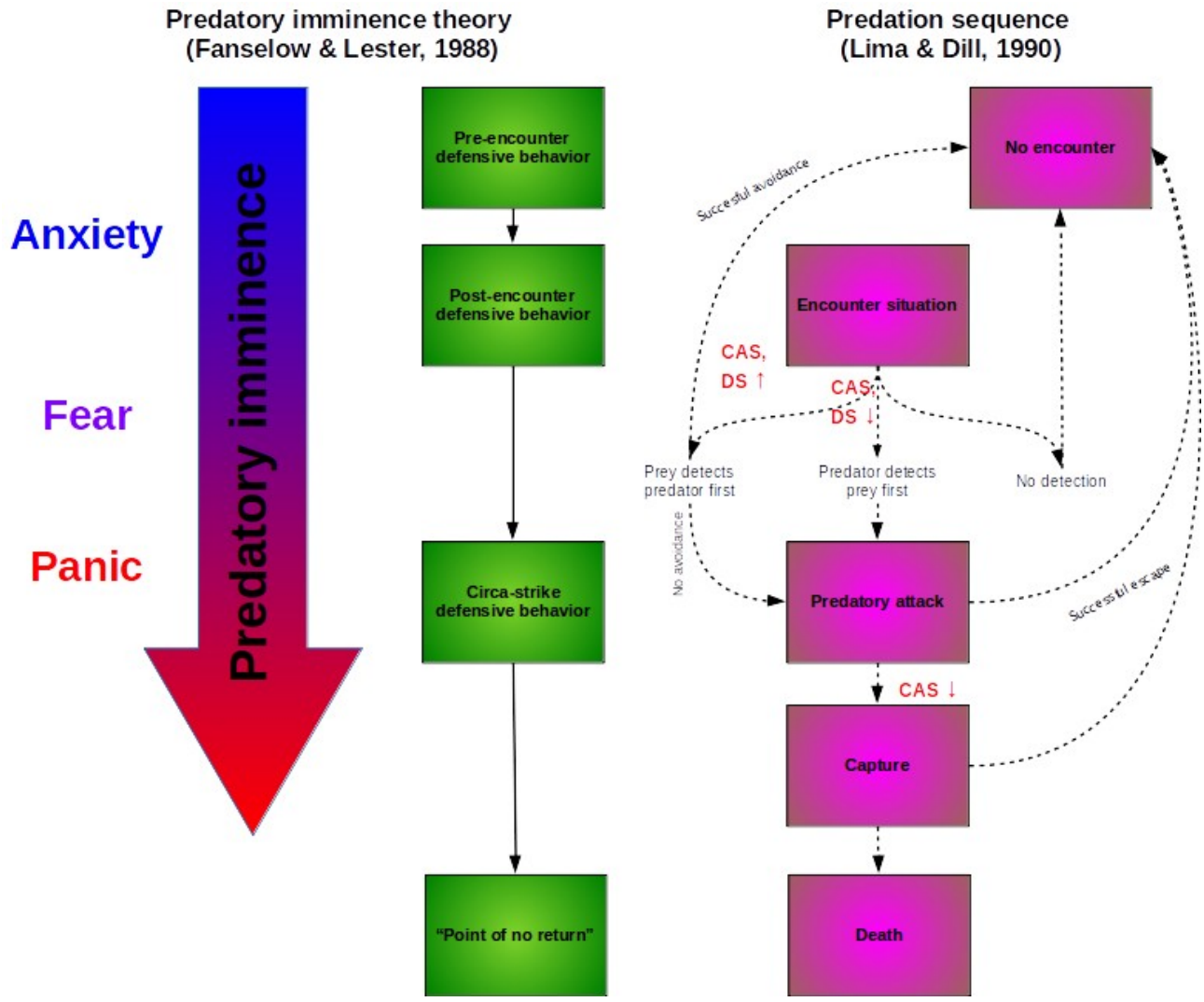

Figure 1 – Alarm (CAS) and disturbance (DS) signals increase post-encounter defensive behavior. On the left the predatory imminence theory of defensive behavior (Fanselow & Lester, 1988) is represented; antipredatory behavior develops from pre-encounter defensive behavior (risk

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assessment behavior) to post-encounter defensive behavior (adjustments to avoid detection) and circa-strike defensive behavior (adjustments to escape or fight the predator); these stages are associated with anxiety, fear, and panic (left arrow; Perusini & Fanselow, 2015). On the right the predator sequence of Lima & Dill (1990) is presented (adapted from Smith, 1992): a prey-predator encounter (i.e., post-encounter situation) develops to either a predatory attack (if either the predator detects the prey first, or if the predator is first detected but avoidance is unsuccessful) or to successful avoidance; a predatory attack (i.e., circa-strike situation) can develop to either capture or successful escape. Conspecific alarm substance or disturbance signals increase the probability of prey detecting the predator first and decrease the probability of the predator detecting the prey first; as a consequence, these signals increase the probability of avoidance. Moreover, according to the predator attraction hypothesis, CAS can also decrease the probability of a successful capture.

CAS is a “partial predator stimulus” (Dielenberg & McGregor, 2006) – that is, it does not faithfully communicates the presence of a predator, since injury could happen from other sources. In rodents, partial predator stimuli induce risk assessment-like responses (i.e., pre-encounter defensive behaviors) instead of altering post-encounter defense (Dielenberg & McGregor, 2006). This is because partial predator stimuli increase uncertainty, since the threat is merely potential (that is, the predator might be or might not be present), and since the direction of the threat is difficult to discern; as a result, careful assessment of the environment is necessary. While CAS certainly fulfills the criteria for a partial predator stimulus at the “signal” side, it does not appear to affect pre-encounter defensive behaviors in fish, instead increasing post-encounter defense (Box 2).

5. Adaptive and evolutionary issues for alarm signals

While it appears clear that CAS is a partial predator stimulus that induces defensive responses (Box 2), there is still some controversy in the field as to what is the evolutionary role of

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CAS. The question was introduced by R. Jan F. Smith in 1992, and has not yet been answered. Two hypothesis are more prominent, the kin selection hypothesis, and the predator attraction hypothesis. In the first case, centered on W. D. Hamilton's theory of kin selection (Hamilton, 1963), since the sender of CAS is paying a high cost because it releases the chemical signal due to potentially mortal damage, the benefits to related individuals (kins) would need to be sufficiently high. Under this hypothesis, CAS benefits kin, and not the sender, because closely related individuals are more likely to share alleles by common descent, and therefore the frequency of the sender's alleles in the next generation would be increased by increasing kin's survival probabilities.

The application of the kin selection hypothesis to the evolution of CAS necessitates at least two assumptions (Smith, 1992): first, there should be evidence that CAS release increases the receivers' fitness; secondly, it should be shown that individuals in a given species associate mainly with kin. There is now ample evidence that CAS increases vigilance, leading to antipredator behavior such as that described in Box 2, as well as long-term alterations in foraging (Oswald & Robison, 2011), fear-induced analgesia (Maximino, 2011; Maximino et al., 2014), and avoidance of areas in which CAS is detected (Chivers & Smith, 1994; Wisenden et al., 1995) or which were previously associated with CAS (Ruhl et al., 2017; Maximino et al., 2018). Guppies (*Poecilia reticulata*) exposed to CAS are more attentive to visual cues (Stephenson, 2016), and zebrafish (*Danio rerio*) exposed to CAS show increased risk assessment in the light/dark test (Quadros et al., 2016; but see Maximino et al., 2014), suggesting that CAS increases alertness to threatening cues in other sensory modalities. On the other hand, CAS was not able to increase antipredator responses to a sympatric predator (although the predator was not in the same tank as the receiver) in zebrafish (Speedie & Gerlai, 2008), although CAS increased survival of fathead minnows (*Pimephales promelas*) placed in an experimental tank with a predator (Mathis & Smith, 1993). Moreover, zebrafish given visual and olfactory access to conspecifics show less freezing when exposed to CAS (Faustino et al., 2017), an effect that has been termed "social buffering". This observation, as well as the fact that

zebrafish tested in groups are less likely to freeze, suggest that conspecific communication is the main function of CAS. Indeed, freezing in a shoal would be maladaptive, since the frozen animal would be more likely to be attacked by the predator than its conspecifics. Therefore, being able to adapt its behavioral response – from freezing to shoal cohesion and erratic swimming – increases the ability of the animal to survive.

The second condition for the kin selection hypothesis of CAS evolution is that individuals should prefer to shoal with kin rather than non-kin. There is mixed support for this hypothesis. For example, there is evidence that zebrafish displays a preference for water in which kin has previously resided over water inhabited by non-kin and unfamiliar kin (Gerlach & Lysiak, 2006). On the other hand, in European minnows (*Phoxinus phoxinus*), no evidence for a preference for kins was found within and between shoals (Naish et al., 1993). Moreover, in fathead minnows the presence of familiar shoalmates is associated with *less* epidermal club cells (Wiseden & Smith, 1998). Glaringly, to the best of our knowledge it is not yet known, in any fish species, whether kin are more responsive to CAS than non-kin, which could increase support to the kin selection hypothesis of CAS evolution.

The competing hypothesis, the predator attraction hypothesis, suggests that CAS attracts additional predators to the area, promoting interactions between the new and the original predator and allowing the sender an opportunity to escape (Smith, 1992). Again, this competing hypothesis carries two important assumptions: the first is that CAS must attract predators; the second is that subsequent predators should disrupt the initial predation encounter, increasing escape probability. Moreover, the sender must be able to recover from the damage in order to escape.

There is some evidence that at least some predator species are able to detect alarm signals, and are actively attracted to them. CAS produced from fathead minnows attracted both predatory fish (*Esox lucius*) and diving beetles (*Colymbetes sculptilis*) (Mathis et al., 1995). Predator attraction to fathead minnow CAS has also been verified in the field, where predators were 7 times more

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likely to strike a lure that was baited with minnow CAS than with water or with skin extract from convict cichlid (*Amatitlania nigrofasciata*)(Wiseden & Thiel, 2002), which presumably do not produce CAS. Thus, at least preliminary support for the first supporting assumption of the predator attraction hypothesis exists.

The second assumption is that additional predators must be able to disrupt predation events in some way, increasing escape probability. It has been shown that the probability of fathead minnows escaping after being captured by a predatory fish is increased by interference by a second pike (Chivers et al., 1996), although the relationship between this event and CAS release has not been investigated. Moreover, only very indirect evidence exists that fish recover from attacks at a high enough probability in order for this hypothesis to be verified; for example, many small fishes in natural populations exhibit scars, presumably from failed predator attempts (Smith & Lemly, 1986). As it stands, there is no strong evidence for either hypothesis on the evolution of CAS.

A third hypothesis of the evolutionary history of CAS is the immune hypothesis. The hypothesis is based on the observation that parasites and pathogens that penetrate the skin of Ostariophysans stimulate the production of club cells (Chivers et al., 2007). This hypothesis states that the primary function of CAS is immune, providing protection against parasites and pathogens, and the sensory ecological ramifications of the substance as an alarm substance evolved subsequently (Chivers et al., 2007). Supporting this hypothesis, skin extracts from fathead minnows, but not from *Xiphophorus helleri* (which are believed to not produce CAS), increased the *in vitro* growth of *Saprolegnia ferax* (Chivers et al., 2007). Moreover, fathead minnows treated with chronic cortisol show reduced club cells in conjunction with reduced leukocyte activity (Halbgewachs et al., 2009). However, a comparative analysis of the roles of CAS on closely-related species – which could clarify the evolutionary history of this trait – is still needed.

6. Mechanisms of CAS detection

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Differently from most vertebrates which communicate via semiochemicals, there is no evidence that Ostariophysan fishes posses a true vomeronasal olfactory system (Eisthen, 1992; Ubeda-Bañon et al., 2011; Maximino et al., 2013). Nonetheless, there is some degree of specialization in olfactory epithelia, as well as in projections of the olfactory system, regarding alarm substances in Ostariophysi (Døving & Lastein, 2009; Bazáes et al., 2013; Kermen et al., 2013).

Odors in the Ostariophysan olfactory epithelium are detected in a combinatorial manner by receptors expressed on olfactory sensory neurons (Kermen et al., 2013). The paired olfactory rosettes, located in the dorsal region of the head near the eyes, contain sensory and non-sensory cells and receptor neurons in the medial region of each lamella. There are three types of olfactory receptor neurons (ORNs) in the Ostariophysan olfactory rosette: ciliated neurons, which have long dendrites and express G-protein coupled odorant receptors; microvillous neurons that express the V2R-like class of receptors; and crypt cells, which express V1R- type receptors (Whitlock, 2006; Oka & Korsching, 2011).

Unlike mammals, in fish a same sensory neuron can express more than one receptor protein (Kermen et al., 2013). Studies using immunohistochemistry have shown that crypt neurons in zebrafish express Gαi proteins (ora4) along with V2R (Oka & Korsching, 2011; Oka et al., 2012), and the detection of the alarm extracts appears to be mediated by crypt cells (Mathuru et al., 2012; De-Carvalho et al., 2013). These cells present a globose morphology and the appearance of both microvilli and cilia within a crypt and form a small agglomeration in the superficial region of the olfactory epithelium (Hansen & Zeiske, 1998). Hamdani & Døving (2002) demonstrated in the crucian carp *Carassius carassius* that the response alarm is mediated by ciliated neurons that project to olfactory bulb (OB), which suggests that in the different species of fish, the detection of the alarm odorants can be mediated by different sensory neurons (Figure 2).

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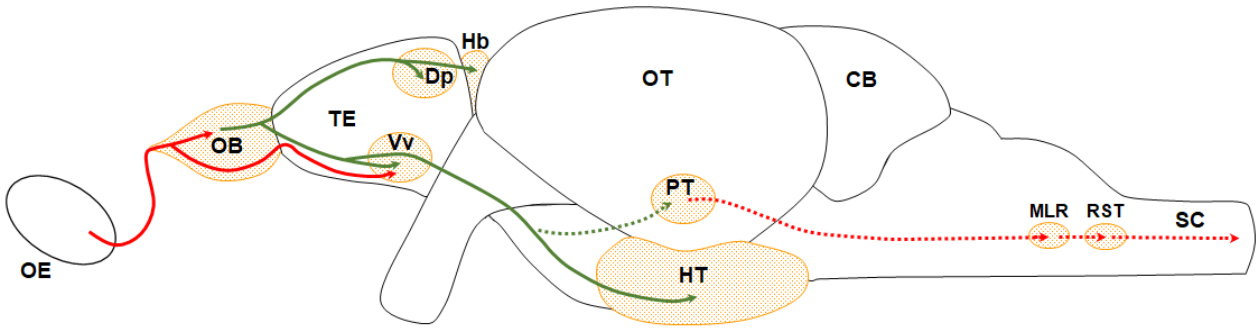

Figure 2 – The olfactory system of teleost fishes. Projections from the olfactory bulb (OB) course through the lateral olfactory tract (LOT) or the medial olfactory tract (MOT). These tracts synapse at the telencephalon (TE) at pallial (posterior zone of the dorsal telencephalon, Dp) and subpallial regions (ventral zone of the ventral telencephalon, Vv); not depicted are MOT terminals at the intermediate (Vi) and postcommisural (Vp) zones of the ventral telencephalon. In the diencephalon, OB projections reach the dorsal habenula (Hb) and the hypothalamus (HT); a putative Vv-posterior tuberculum (PT) projection is also depicted (dashed green arrow), which gives rise to a motor pathway that ends in the mesencephalic locomotor region (MLR) and reticulospinal tract (RST). Vv also receives a direct projection from the olfactory epithelium (red arrow). *Abbreviations:* OE: Olfactory epithelium; OB: Olfactory bulb; TE: telencephalon; Dp: posterior zone of the dorsal telencephalon; Vv: ventral zone of the ventral telencephalon; Hb: Habenula; OT: optic tectum; PT: posterior tuberculum; HT: Hypothalamus; CB: cerebellum; MLR: mesencephalic locomotor region; RST: reticulospinal tract; SC: spinal cord. Based on Kermen et al., (2013).

ORNs have fine axons that terminate in the olfactory bulb in specific synaptic structures known as glomeruli (Braubach et al., 2012), which contact dendrites of mitral cells, the output cell of the olfactory bulb (Kermen et al., 2013). The projection from the olfactory epithelium segregates at different targets in the olfactory bulb (OB); in zebrafish, for example, it has been shown that ciliated cells preferentially innervate the dorsal and anteromedial glomerular fields (Gayoso et al.,

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2012), while crypt ORNs project mainly towards the dorsomedial field (Sato et al., 2005; Gayoso et al., 2011, 2012). A subset of crypt cells respond to kin odors in zebrafish (Biechl et al., 2017), Mathuru et al (2012) demonstrated, through wide-field fluorescence microscopy, that pattern of zebrafish OB activation by partially purified skin extracts involve three distinct loci, located in the anterior plexus, the lateral chain, and the mediodorsal posterior bulb. Interestingly, neurons in the dorsomedial field that project to the habenula, an important structure in the mediation of behavioral responses to CAS (see below), do *not* show increased *fos* expression after alarm substance (DeCarvalho et al., 2013). In crucian carp, crypt cells project preferentially towards the ventral OB (Hamdani & Døving, 2006); nonetheless, single unit responses to CAS were observed mainly in the dorsomedial field as well (Lastein et al., 2008). Extracellular recordings of nervous activity of units composed of mitral cells in alarm region of OB concomitant stimulation at the olfactory epithelium by skin extracts showed that several of these units respond to and discriminate between conspecific and heterospecific skin extracts (Lastein et al., 2008). However, although the number of units activated was more elevated when stimuli were applied in high concentrations, at low concentrations, the units in the alarm region showed increased discrimination between conspecific and heterospecific skin extracts (Lastein et al., 2008). In this species, CAS elicited an increase in the firing of "type I" cells in the medial field (Hamdani & Døving, 2003), which are characterized by a diphasic action potential with a relatively small amplitude, a short duration, and high spontaneous activity (Hamdani & Døving, 2003).

The mitral cells of the OB extend their axons through the medial (MOT) and lateral olfactory tracts (LOT) to different higher brain centers; in carp and zebrafish, the LOT contains mainly fibers originating in lateral domains of the OB, while the MOT contains mainly fibers originating in medial domains (Kermen et al., 2013). The teleost MOT is subdivided into medial and lateral regions (Kermen et al., 2013). In crucian carp, lesions of the medial bundle of the MOT, leaving the

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lateral bundles intact, abolish alarm reactions to skin extract, demonstrating the specificity of the spatial aspect of olfactory processing (Hamdani et al., 2000).

OB projections reach mainly the posterior zone of dorsal telencephalic area (Dp), the ventral zone of the ventral telencephalic area (Vv), the medial comparment of the right habenula (MdHb), the posterior tuberculum (PT), and the hypothalamus (Hyp), with secondary projections from the PT to the mesencephalon and reticulo-spinal motor nuclei (Kermen et al., 2013). After entering the telencephalon, the zebrafish MOT initially runs laterally to Vv and then rises laterally to the dorsal zone of the ventral telecephalic area (Vd) up to commissural levels (Biechl et al., 2017). At postcommissural levels, the MOT forms a large terminal field covering the postcommissural zone of the ventral telencephalic area (Vp) and the intermediate zone of the ventral telencephalic area (Vi) (Biechl et al., 2017). The participation of these regions, as well as their projections, in the alarm reaction will be the topic of the next section.

8. Neural bases of the alarm reaction

After detection and initial processing by the olfactory system, CAS initiates an alarm reaction that can be mediated by other structures. Using the expression of *cfos*, an immediate early gene, some regions which were activated by CAS were identified in zebrafish (Faustino et al., 2017; Ruhl et al., 2017), and include the medial zone of the dorsal telencephalon (Dm, homologous to the mammalian basolateral amygdala [Maximino et al., 2013]), Vv, Vp, and preoptic area (POA, partially homologous to the mammalian hypothalamic paraventricular nucleus [Goodson & Kingsbury, 2013]). These regions are part of a brain aversive system (Figure 3) that detects aversive stimuli and integrates neurobehavioral responses in fish. Interestingly, while these regions were activated by CAS exposure in zebrafish, the correlation between them *decreased* (Faustino et al., 2017), suggesting that inhibitory connections are important for the regulation of the activity of these regions.

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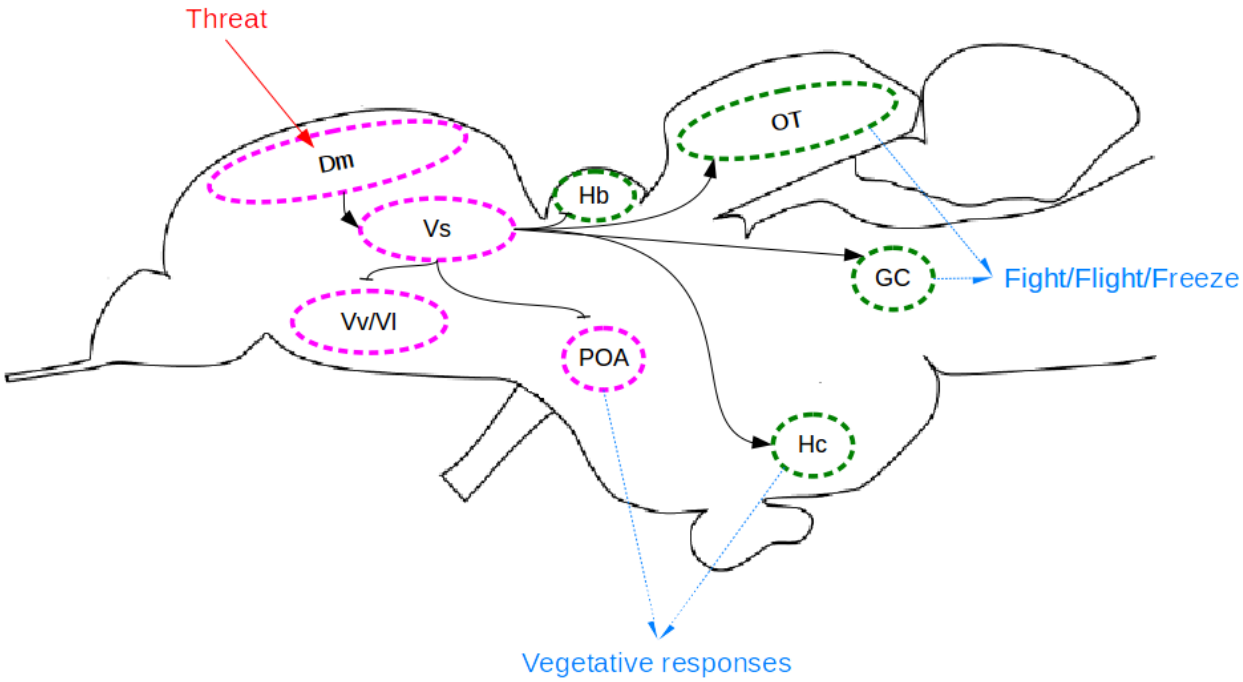

346 **Figure 3 – The brain aversive system of teleosts.** Structures involved in threat detection and ap-
347 praisal are shown, including structures which have been shown to be activated by CAS in zebrafish
348 (Faustino et al., 2017; Ruhl et al., 2017). The main circuit in the interpretation of threatening stimu-
349 lus is Dm, the homologue of the mammalian basolateral amygdala. This structure projects to the
350 precommissural (Vs) and supracommissural (Vp) zones of the ventral telecephalon, which project
351 (putatively inhibitory) axons to the ventral (Vv), intermediate (Vi), and lateral (Vl) zones of the
352 ventral telencephalon, to the habenula (Hb), and to the preoptic area (POA), as well as projections
353 to the caudal hypothalamus (Hc), optic tectum (OT), and central gray (GC). The POA and the Hc
354 generate the vegetative outputs of CAS (cortisol and norepinephrine/epinephrine release), while OT
355 and GC generate the behavioral outputs (fight/flight/freeze). Structures shown to be activated by
356 CAS are identified in pink.

357

358 These regions receive primary or secondary projections from the central olfactory system
359 (Folgueira et al., 2004; Miyasaka et al., 2009; Gayoso et al., 2011). Vv (the putative homologue of
360 the striatum [O'Connell & Hofmann, 2011; Goodson & Kingsbury, 2013]) and Vp (a partial homo-
361 logue of the central amygdala [Maximino et al., 2013]) receive direct projections from the OB via

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MOT (Biechl et al., 2017). Interestingly, an olfactory projection from the OB to the medial zone of the dorsal right habenula (dHbM) has been described in zebrafish (Miyasaka et al., 2009), but the OB zones which originate this projection are *not* activated by CAS or chondroitin sulfate (DeCarvalho et al., 2013). Likewise, DeCarvalho et al. (2013) did not identify *cfos* expression in any zone of the habenula after CAS or chondroitin sulfate exposition.

This lack of habenular activity is a paradox, given the observation of a role of this structure in organizing olfactory-driven behaviors (Krishnan et al., 2014) and its role in regulating aversive behaviors in zebrafish (Agetsuma et al., 2010; Okamoto et al., 2011; Amo et al., 2014; Chou et al., 2016). The habenular complex is a paired structure found in the diencephalon of all vertebrates, connecting the forebrain to the midbrain. Teleostean habenulas are asymmetric and may contribute to lateralized behavior. The dorsal zone can be subdivided into asymmetric subnuclei, based on their different molecular properties (Halpern et al., 2003; Okamoto et al., 2011). Silencing the dHb in adult zebrafish increased the response to a low CAS concentration (Mathuru & Jesuthasan, 2013). A dHbL-intermediate interpeduncular nucleus-central gray pathway switches behavior between offensive and defensive behavior, while a dHbM-ventral interpeduncular nucleus-median raphe nucleus (MRN) pathway controls serotonin release and resilience to aversive stimuli (Amo et al., 2014; Chou et al., 2016). The ventral habenula (vHb), on the other hand, does not appear to be lateralized in zebrafish, and tonic responses in the zebrafish vHb represent an aversive expectation value, participating in a larger vHb-MRN circuit (Amo et al., 2014).

In zebrafish, *kiss1* and the *kissr1* receptor are predominantly expressed in the vHb (Ogawa et al., 2012; Ogawa & Parhar, 2013; Nathan et al., 2015b). vHb projects Kiss1-expressing neuronal fibers to the MRN (Servili et al., 2011; Ogawa et al., 2012; Nathan et al., 2015b), one of the important conserved serotonergic (5-HTergic) nuclei in the zebrafish (Lillesaar, 2011). Intracerebral administration of Kiss1 significantly reduced the freezing and erratic swimming behaviors evoked by CAS; however, injection of kisspeptin conjugated with saporin (to selectively inactivate Kiss-R1-

expressing neurons) decreases non-stimulated *cfos* activity in both vHb and MRN, and also abolishes the behavioral effects of CAS (Ogawa et al., 2014). This suggests that kiss1 decreases responsiveness to CAS in vHb targets such as the MRN, but increase it (via Kiss-R1) in the vHb. Indeed, chronic (8 days) exposure to CAS reduced the expression of *kiss1* in the zebrafish brain, as well as genes associated with 5-HTergic signaling (Ogawa et al., 2014).

Strengthening the hypothesis of a mediation by the serotonergic system is the observation that CAS-induced geotaxis is blocked by kiss1, and that this effect is itself blocked by both 5-HT_{1A} and 5-HT_2 receptor antagonists in zebrafish (Nathan et al., 2015a). Interestingly, methysergide, the 5-HT_2 antagonist, was also able to block the effects of Kiss1 on CAS-induced freezing, an effect which was not observed with the 5-HT_{1A} receptor antagonist WAY 100,635 (Nathan et al., 2015a). Moreover, both drugs dose-dependently increased the effects of CAS on geotaxis and freezing, but methysergide produced effects at all doses, while WAY 100,635 only produced an effect at a very high dose (Nathan et al., 2015a). Lower doses of WAY 100,635 were unable to block the post-stimulation effects of CAS on the light/dark test, and did not block the CAS-elicited sympathetic activation, but were able to block the antinociceptive effect of CAS (Maximino et al., 2014); on the other hand, acute fluoxetine was able to block post-stimulation effects of CAS on the light/dark test and the sympathoactivation, but not the antinociceptive effect (Maximino et al., 2014).

The activation of the POA that is observed after CAS exposure is possibly related to the neuroendocrine profile that is observed in CAS-exposed animals. Increases in cortisol levels were observed after CAS (Mathuru et al., 2012; Schirmer et al., 2013; Silva et al., 2015; Abreu et al., 2017) and disturbance signals (Barcellos et al., 2011, 2014, Oliveira et al., 2013, 2017; see Box 2) in different species. Moreover, in zebrafish CAS elevates plasma levels of norepinephrine, epinephrine, and glucose (Maximino et al., 2014), strongly implicating the sympathetic system in these vegetative adjustments. In spite of these observations, a causal relationship between cortisol and/or plasmatic catecholamines and the alarm reaction has not, so far, been established.

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A limitation in the approaches to describing the circuitry involved in the alarm reaction is that they purposefuly analyzed only a handful of regions to increase power; as such, there are many structures which have not been analyzed, but which interact with this “core circuit” (Figure 3). The main region in the interpretation of threatening stimulus is the Dm, the homologue of the mammalian basolateral amygdala. This structure projects to the precommissural (Vs) and supracommissural (Vp) zones of the ventral telecephalon, which by its turn project (putatively inhibitory) axons to the ventral (Vv), intermediate (Vi), and lateral (Vl) zones of the ventral telencephalon, to the habenula (Hb), and to the preoptic area (POA). Other projections from Vs include the caudal hypothalamus (Hc), optic tectum (OT), and central gray (GC). The POA and the Hc generate the vegetative outputs of CAS (cortisol and norepinephrine/epinephrine release), while OT and GC generate the behavioral outputs (fight/flight/freeze). The inhibitory projection to the habenula could explain the failure to find *cfos* activation in this region in spite of its participation in regulating the alarm reaction. As a result of this inhibition, vHb-MRN tonus would decrease, leading to a reorganization of behavior towards risk assessment and freezing.

Other neurochemical systems have also been implicated in the alarm reaction. In zebrafish, CAS reduces AMP hydrolysis, an important source of adenosine in the brain (Canzian et al., 2017). Since adenosine appears to exert an anxiolytic-like effect via the A1 and A3 receptors (Maximino et al., 2011, 2015), a down-regulation of this pathway could represent an important mechanism of anxiogenesis. Finally, THC has been shown to impair the acquisition of a conditioned fear response to a visual stimulus that was paired with CAS on zebrafish, and also attenuated the behavioral responses during CAS exposure (Ruhl et al., 2017). Interestingly, THC treatment also reduced CAS-elicited c-Fos expression in the Dm and Dl after conditioning in these animals (Ruhl et al., 2017), suggesting that cannabinoids impair stimulus encoding in these pallial areas. However, whether endocannabinoids participate in the organization of alarm reactions is so far unknown.

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9. Applications of the alarm reaction: Models for panic disorder and PTSD in zebrafish

The observation of the different behavioral and neuroendocrine effects of CAS suggest its use as a stressful stimulus in modeling threat- and stress-related disorders in fish. Stress reactions and reactions to acute threat are related to several factors, not just those caused by the aggression by other bodies and physical agencies, but also the consequences to man's ability to interpret syndromes as indications of danger derived from their past experience (Weiss, 1968; Coppens et al., 2010; Koolhaas et al., 2010).

Given that CAS is a partial predator stimulus that signals a potentially life-threatening situation and induces sympathetic (Maximino et al., 2014) and corticosteroid activity (Abreu et al., 2017), it is possible that long-term changes in behavior after CAS exposure could be used to model post-traumatic stress disorder (PTSD). PTSD presents two central features: exposure to an event that involves life-threatening or serious injury to themselves or others, linked to intense fear, despair, or horror (Olff et al., 2005; Miller & McEwen, 2006; Rao et al., 2009). In response to this traumatic event, some symptoms are developed, such as flashbacks of the traumatic event, avoidance of stimuli associated to the event, hypervigilance, and hyperexcitability (Figueira & Mendlowicz, 2003). Importantly, PTSD is defined as a *delayed* response to the life-threatening situation, since, in order to be diagnosed with PTSD the individual needs to experience symptoms at least 6 months after the traumatic event (American Psychiatric Association, 2013). The frequency and the degree to which an individual is anxious or afraid are extremely important for the diagnosis of certain psychiatric disorders.

Currently, rodent models for PTSD are based on the animal's exposure to extreme stress situations, resulting in intense fear responses in the animal (Matar et al., 2013). After exposure to a stressful protocol, usually involving the presentation of a predator or partial predator stimulus, the animal displays behavioral characteristics that are similar to those found in PTSD, including increased anxiety- or depressive-like behavior (Cohen et al., 2011; Matar et al., 2013).

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In zebrafish, the use of prolonged exposure to a predator has been proposed as a model for PTSD (Stewart et al., 2014a); while this setup produces protracted predator avoidance, it is not able to model situations in which the traumatic event is brief, a requirement for adequate models for PTSD (Yehuda & Antelman, 1993). We have exploited CAS as a stimulus to induce a PTSD-like syndrome in zebrafish (Lima et al., 2015, 2016). PTSD produced anxiety-like behavior that is qualitatively and quantitatively different 24 h after exposure than immediately after exposure, suggesting that the stress-free period leads to incubation of stress/fear responses instead of merely producing a sustained response (Lima et al., 2016). Moreover, the sensitization was observed in two different tests for anxiety-like behavior, as well as in startle responses, modeling two domains (anxiety and hypervigilance) that are altered in PTSD (Lima et al., 2016). Finally, this time-dependent sensitization does not affect all animals equally, with about one-quarter of the animals showing maladaptive responses, and another quarter displaying resilience (Lima et al., 2016). Currently, the model is being used to evaluate the role of nitric oxide on PTSD (see also Lima et al., 2015).

Recently, the observation that zebrafish CAS produces different behavioral phenotypes during and after exposure (Box 2) led us to propose using these responses as models for panic attacks and panic disorder, respectively (Silva et al., 2018). Tracing a parallel with two types of freezing evoked by electrical stimulation of the dorsal periaqueductal gray area (dPAG) of rats – dPAG-evoked freezing and post-stimulation freezing (Brandão et al., 2008) – we proposed that the increase in erratic swimming that is consistently observed during CAS exposure (Box 2) is an escape (panic-like) response, while the increase in freezing that is observed post-exposure is more associated with risk assessment (anxiety-like, allowing the animal to evaluate the consequences of the aversive stimulus). This model is currently being used to evaluate the role of the serotonergic system on both responses.

10. Conclusions

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Our current knowledge on alarm substances and disturbance signals increased considerably since Pfeiffer (1977) described its distribution and Smith (1992) suggested hypotheses for its evolution. It is now known that alarm substances evolved independently in other fish species, and that can be independent from club cells, raising the question of alternative mechanisms for CAS production. While most of the focus in the past has been on the ecological consequences of CAS and its adaptive functions, research in the last 20 years focused mainly on the neural basis of CAS detection and alarm reaction generation. These two research traditions rarely cross-fertilized each other; however, good ethological validation, ecological relevance, and adequate knowledge of the neural bases of a given behavioral function is crucial for its use as a model system in the behavioral neurosciences and neuropharmacology (van der Staay, 2006; Maximino, 2017). As a result – and as a consequence of the ascension of zebrafish as a model organism in the field (Kalueff et al., 2014; Stewart et al., 2014b; Shams et al., 2018) – more focus has been given on the applications of CAS as an aversive stimulus in many different paradigms, from aversive conditioning (Ruhl et al., 2017; Maximino et al., 2018) to behavioral models in psychopathology (see Box 2).

Many important research questions remain. What, if any, is the functional significance of species differences in stimulus detection? Given that CAS induce sympathetic activity and glucocorticoid release, what are the effects of alarm substances (and disturbance signals) on metabolism (e.g., glucose production, osmoregulation, oxidative metabolism)? What is the role of environmental niches (e.g., substrate, water turbidity, water flow velocity) on the behavioral effects of CAS? How specific mediators (serotonin, glucocorticoids, catecholamines) produce variation in these responses? Is there a relationship between the environment in which a species evolved CAS and variation in these neural systems? These are relevant gaps in the literature that await further investigation.

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