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Fish models in neural and behavioral toxicology: Expanding beyond mortality and teratogenicity

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Abstract

The industry is increasingly relying on fish for toxicity assessment. However, current guidelines for toxicity assessment focus on teratogenicity and mortality. From an ecotoxicological point of view, however, these endpoints are not sensitive enough, as they are not able to detect sub-lethal or non-teratogenic effects that can nonetheless result in decreased fitness and/or inability to adapt to a changing environment, affecting whole populations. Impacts of toxicants on neurobehavioral function have the potential to affect many different life-history traits, and are easier to assess in the laboratory than in the wild. We propose that carefully-controlled laboratory experiments on different behavioral domains – including anxiety, aggression, and exploration – can increase our understanding of the ecotoxicological impacts of contaminants, since these domains are related to traits such as defense, sociality, and reproduction, directly impacting life-history traits. We review the effects of selected contaminants on these tests, focusing on larval and adult zebrafish, showing that these behavioral domains are highly sensitive to small concentrations of these substances. These strategies suggest a way forward on ecotoxicological research using fish.

Keywords: Neurobehavioral assessment; Ecotoxicology; Zebrafish; Neurotoxicology

1. Introduction

Ecotoxicology, as a field, currently experiences an influx of research using fish as subjects, in part due to increased interest in the toxicology of pesticides and waste from

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pharmaceutical products. While ecotoxicology usually focuses on toxic effects at the population, community, ecosystem, and biosphere levels, the integrative approach usually demanded to reach this level of analysis implicates the effects of toxicants across all levels of biological organization [1]. As a result, all effects which are likely to result in decreased fitness and/or inability to adapt to a changing environment are of relevance. Clearly, impacts of toxicants on neurobehavioral function have the potential to impact on many different domains of life [2,3]. Consider, for example, effects on defensive behavior: contamination which changes the appropriate levels of antipredator defense and cautious exploratory behavior can lead to decreased ability to escape or avoid actual or potential threats, causing either death or the loss of important resources, such as access to mates or food.

The field of ecotoxicological research using fish species gained much traction in the last 20 years [4]. Pressures from special interests groups, as well as increasing awareness from regulatory agencies that lethal endpoints are not appropriate for ecotoxicology, have galvanized research in the field in a way that sublethal behavioral and physiological effects are now more common in the field than the usual protocols. OECD guidelines for assessing the toxicity of chemicals in fish include protocols with lethal endpoints (for acute toxicity in adults [OECD 203], embryo toxicity [OECD 210 and 236], toxicity tests on egg and sac-fry stages [OECD 210 and 212]), as well as sublethal endpoints (larval and juvenile growth [OECD 210 and 215], sexual and endocrine development [OECD 230 and 234]). However, the field as a whole is rapidly moving beyond mortality and teratogenicity. The present paper reviews selected references in the field of neurobehavioral ecotoxicology research, proposing the use of a handful of sensitive and ecologically relevant behavioral assays to expand beyond lethal and/or crude morphological endpoints. We begin by dispelling confusions on the use of fish as both model and target species in ecotoxicological research. We proceed by discussing acute toxicity tests that are used in adult animals, briefly discussing OECD protocols and fish acute toxicity syndromes (FATS). We briefly discuss the widely-used fish embryo test (FET) before moving on to the core

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of the article, biobehavioral assays in neurotoxicology.

2. Using fish as models and targets in ecotoxicology

Fish are directly affected by a plethora of environmental toxicants, including heavy metals from mining, pesticides, and pharmaceutical waste from human consumption. Studying the effects of a contaminant on fish behavior with the objective of understanding impacts on fish populations is important in that case, as fish are the target species - that is, information regarding the neurobehavioral toxicology of a putative contaminant is not used to infer effects on other species, but in the test species itself. In that case, wild species are best suited, preferably derived from wild populations from areas that are contaminated.

In many cases, however, fish are used as *model organisms* - that is, as a “surrogate species” that substitutes another species in experimentation and is therefore being studied primarily not because they are interesting in their own right (though they may well be), but because of the value they can have for investigating effects that can be generalised [5]. Model organisms target a wide range of systems and processes occurring in living organisms, including genetics, development, physiology, behavior, evolution, and ecology [6,7]. Usually, model organisms are selected based on “experimental characteristics that closely relate to their power as genetic tools” [7]: small sizes; low costs to breed, maintain and transport; short generation times and life cycles; high fertility rates; high susceptibility to techniques for genetic modification. In addition to that, infrastructure (including shared databases and tools) and a well-established community of researchers that rely on the species as a model organism are also fundamental [7].

Fish can be used as model organisms in ecotoxicology in at least two important contexts: the first is of using a fish as a model organism to infer processes and effects on other

fish species. For example, organic mercury intoxication in the Amazon, due to bioaccumulation, highly impacts piscivorous fish [8] that are hard to raise in laboratory contexts, and to which neurogenomic and behavioral tools are unavailable. Using smaller fish species - including well-established model organisms such as zebrafish and medaka [9] - can circumvent these difficulties if we assume that the physiology of teleost fish is relatively well-conserved across major taxa.

The second important context of using fish as model organisms is that of when the target species is a mammalian, including humans. In many well-established protocols for teratogenicity and reproductive toxicology, the implicit assumption appears to be that, if a given substance is teratogenic in fish embryos, one can assume that it will also be in human embryos. While the physiological distance between fish and humans is certainly higher than from teleost to teleost, or even to non-teleosts, the usual assumption is that there are enough commonalities to facilitate extrapolation [4,9,10]. Moreover, from a mechanistic and evolutionary point of view, the discovery of a mechanism of toxicity that is shared between non-human mammals and fish make it more likely that the mechanism is evolutionarily conserved, and therefore also shared with humans [11].

3. Acute toxicity tests in adults and larvae

3.1. OECD adult protocols

The acute toxicity test (OECD 203) is an updated protocol applied in acute toxicity testing in fish [12]. Compared to previous versions, the main changes are the reduction of sample sizes, the extension in the concentration range, and the introduction of a limit test. The choice of the use of one or more fish species is in accordance with the test

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laboratory criteria. The main endpoint of the protocol is mortality; individuals (up to seven fish per group) are exposed to a test substance, dispersed in the water (waterborne administration) for 96 hours, and mortalities are recorded at 24, 48, 72, and 96 hours post-exposure. At least five concentrations, in a geometric series with a factor not exceeding 2.2, should be used. When an experiment results in at least two concentrations with partial mortalities, the lethal concentration (LC_{50}), the confidence limits (95%), and the slope of the curve should be estimated using classical maximum likelihood methods for fitting probit or logit models; however, when only one concentration produces partial mortality, estimates of the LC_{50} can be made using various techniques such as the Spearman-Karber method, the binomial method, the moving average method, or the graphical method (a last resort). A proposed update of OECD 203 also includes sublethal signs that the fish may display during toxicity testing. These are shown in Table 1.

The validity of the acute toxicity test is dependent on determining whether the cause of the elevated mortality is indeed the tested substance. For that, mortality in the control group(s) should not exceed 10% at the end of the test. Moreover, dissolved oxygen concentration must have been at least 60% of air saturation value throughout the test, and therefore dissolved oxygen should be monitored.

Domain	**Clinical sign**
Distribution	Loss or densing of schooling / shoaling behavior
	Vertical distribution - Surfacing or bottom-dwelling
Equilibrium and	Abnormal horizontal orientation
	Abnormal vertical orientation

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buoyancy	Loss of buoyancy control
Observed behaviors	Hypoactivity or hyperactivity
	Spiral swimming
	Hyperventilation or hypoventilation
	Irregular ventilation
	Increased ventilation depth
	Convulsions
	Coughing, gulping, or gasping
	Surface escape / avoidance behaviors
	Bottom escape / avoidance behaviors
	Irritated skin behaviors
	Aggression and/or cannibalism
Appearance	Tetany
	Skin color - Darkening, lightening, or mottled skin
	Oedema
	Hemorrhagic areas or petechiae
	Exophthalmia
	Mucus secretion
	Faecal casts
Provoked behavior	Visual and tank knocking stimulus - over reactivity or under reactivity
	Tactile stimulus - over reactivity or under reactivity

3.2. Fish acute toxicity syndromes (FATS) assays

OECD 203 is very limited in terms of both ecotoxicology and neurotoxicology because its endpoint is lethality; even if sublethal signs are observed, there is no systematic way to evaluate these, and the significance of these signs to the behavioral

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ecology of the animal is sometimes limited. The fish acute toxicity syndromes (FATS) paradigm has been introduced to systematically evaluate other parameters besides mortality.

This protocol is not performed in experiments with Zebrafish, but is done in other species of fish; in its first versions, FATS was applied to Rainbow trout (*Onchorhynchus mykiss*) [13,14]. Fish undergo surgery prior to exposure to toxicants to implant respiratory and cardiovascular monitoring devices. In the first experiments, exposure was made from 24 to 48 h [13–15], resulting in acute toxicity. Endpoints include cough rate, ventilation rate, ventilation volume, total oxygen consumption, oxygen utilization, heart rate, arterial blood pressure, arterial blood oxygen, arterial blood carbon dioxide, arterial blood pH, hematocrit, hemoglobin, electrocardiogram, plasma ions (calcium, magnesium, potassium, sodium, and chloride), and osmolality [13].

The main difference compared to other acute toxicity protocols is the objective of understanding the mechanisms of action of test substances. The understanding of the mechanism is not only determined to represent particular events at the molecular level, but also to understand the causal function in specific toxin, for this there are direct and indirect measures of the biological response of the organism produced for each substance. For example, the combination of quantitative structure-activity relationship (QSAR) information with the effects of toxicants on FATS endpoints is a promising avenue that points to FATS as an interesting toxicological screen [16–18]. These qualities suggest that FATS assays have good predictive validity - that is, they are capable of discriminating between different toxic effects based on mechanism of action.

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3.3. The fish embryo test

The fish embryo toxicity test (FET) used to determine the toxicological toxicological action of a drug. Doses are applied at different stages of the zebrafish (*Danio rerio*) embryo. The fish were fertilized, the eggs collected and exposed to the chemical test for a period of 96 hours. After the application, observations are made on the effect of the lethality of the drug under the embryos.

4. Behavioral bioassays in neurotoxicology

Acute toxicity assays, whether using lethal or sublethal endpoints, usually focus on dramatic effects which lead to a very high lethality. However, as we argued above, there are many toxic effects that are relevant for ecotoxicology [2,19]. Behavior can be used as an assay of fitness [20], given its importance to the viability of the organism, the population, and the community. Peterson et al. [19] pointed that toxicants can produce effects at individual-level responses, which in its turn impact responses at the population and higher (species, community, ecosystem, and evolutionary) levels (Fig. 1).

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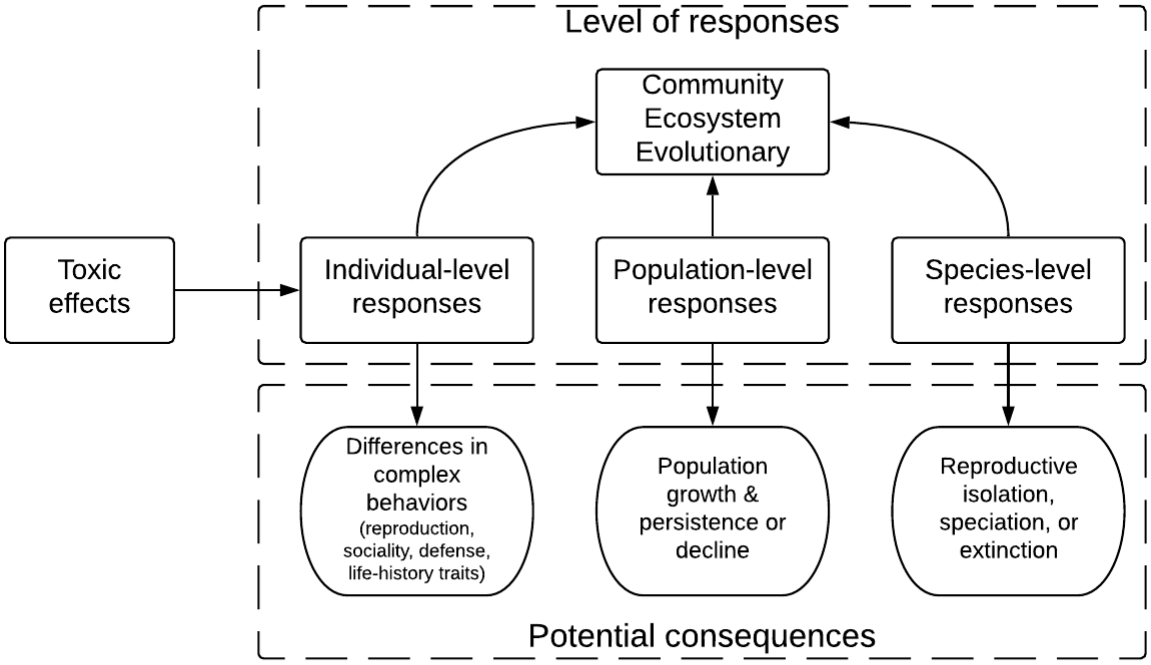

There are several advantages of incorporating behavior in ecotoxicology [19]:

1. behavior is an indicator of multiple levels of biological effects
2. behavior is among the most sensitive indicators of impact of exposure (10-1,000 times more sensitive than lethality measures)
3. behavior is considered an early warning tool.

One difficulty of incorporating behavior, however, is that of ecological validity. In psychometry, ecological validity is a measure of how test performance predicts behaviors in real-world settings; in the case at hand, the highest possible ecological validity is observing behavior in the wild. This is, in many cases, impractical, and does not lend itself to mechanistic ecotoxicology, as analyzing the effects of single toxicants, or knowing precise concentrations, is impossible, and avoiding contamination is very difficult.

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The solution is to develop behavioral bioassays. “behavioral bioassays measure an organism’s behavior, qualitatively or quantitatively, to detect and analyze some external stimulus or as an indicator of an internal physiological or psychological state” [21]. In the literature, most assays are described as related to behaviors that are associated with mental disorders - anxiety-like behavior, aggressive behavior, compulsive-like behavior; however, these assays allow broader comprehension of a mechanism, without necessary causal analogy with the etiology or pathological basis of the mental disorder [22]. As a result, behavioral bioassays are used to study normal behavior and/or the effects of perturbations on this behavior [23].

In fact, most of the so-called “animal models” in fish research [24] are not models *per se*, but screening tests or behavioral bioassays [22]. These models usually present good predictive validity, in that they discriminate between drugs with clinical efficacy [25]. The predictive validity of most of these assays to discriminate between toxic effects and/or mechanism of action, however, is not yet established. This represents both an opportunity and a difficulty for research: part of the necessary steps in introducing behavioral bioassays in aquatic ecotoxicology involves establishing predictive validity and conducting mechanistic research for different assays - i.e., establishing how specific toxicants induce predictable neurobehavioral and physiological responses at different levels.

In addition to using behavioral assays which directly mimic challenges faced in the wild, such as conspecific conflict, foraging, and antipredator defense, it can be useful to assess behavior in assays which carefully target the neurobehavioral domains that are recruited during these challenges. This is the “ethoexperimental” approach [26]

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that we will follow in this review. For zebrafish and other small teleost species, there are currently well-validated assays to study sociality [27], anxiety-like behavior [28], aggression [29], and fear [30], as well as appetitive learning [31], which is relevant to foraging (Fig. 2). While ecological validity is diminished by analysing behavior at a more "molecular" level of a "molar", composite function, this is offset by greater experimental control and ability to manipulate and test stimulus control.

In the remainder of this review, we will focus on these behavioral assays and how they have been used in studying the effects of toxicants. Specific focus will be given to locomotor activity, which is relevant to many different domains, including defensive behavior, foraging, and habitat use; fear and anxiety, which is relevant for antipredator defense; and aggression, which is relevant to defense against conspecifics and territoriality.

4.1. Locomotor activity assays

4.1.1. Larvae

Locomotor activity is sensitive to many different perturbations, and is organized at different levels of the nervous system. Alterations in locomotor activity, therefore, can represent alterations at the neuromuscular junction, at the muscle, at central pattern generators in the spinal cord or in the hindbrain, or in more rostral levels. While highly sensitive to perturbations, usually assays for locomotor activity focuses on distance or speed, which are not very selective.

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Fish larvae show greater activity during dark cycles than during light cycles; thus, larvae respond to changes in illumination with both acute responses and extended behavioral responses [32,33]. A wide range of compounds have been tested in locomotion of zebrafish larvae, and its acute effect be classified crudely as sedative-like or stimulant-like (Table 2). As can be deprehended from the table, if one analyses only simple endpoints, such as distance travelled or swim speed, predictive validity is very low - for example, cocaine and amphetamine clearly do not produce sedative-like effects in other animals.

Class	**Compound**	**Concentration range**	**Age**	**Ref.**
Sedative-like	4-aminopyridine	0.6 mM	5 dpf	[34]
	Clozapine	12.5 - 50 mM	7 dpf	[35]
	Cocaine	0.2 - 50 µM	6 dpf	[36]
	Amphetamine	0.1 - 20 µM	6 dpf	[36]
	Diazepam	10 - 100 nM	7-14 dpf	[36]
	Fluoxetine	4.6 mM	3-6 dpf	[37]
	Polybrominated diphenyl ether (DE-71)	31 µg/L	5 dpf	[38]
	Graphene quantum dots (light period)	12.5-200 mg/L	4 hpf - 96 hpf	[39]
	Silica nanoparticles (dark period)	25 and 50 mg/L	4 hpf - 96 hpf	[40]
	Perfluorooctanesulphonic acid (PFOS)	0 - 8 mg/L	6 hpf - 120 hpf	[41]
Stimulant-like	Silica nanoparticles (dark period)	100 and 200 µg/mL	4 hpf - 96 hpf	[40]

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	4-Aminopyiridine	0.8 - 2.5 mM	5	[34]
	Aconitine	2.5 - 25 µM	5	[34]
	Bisphenol A	0.01 - 1 µM	5	[42]
	Pentylenetetrazole	10 mM	5	[34]

As a result of this lack of specificity in locomotor activity assays in fish larvae, many different tests have been developed, mainly with zebrafish in focus [33]. Zebrafish larvae exhibit different behaviors to different stimuli which can be exploited to assess different neurobehavioral domains (Table 3). These responses allow a much more detailed investigation of locomotor behavior at earlier life stages and with ecologically-relevant endpoints.

behavior	**Age obs.**	**Stimulus**	**Example compound**
Coiling	17-21 hpf	None	PFOS [41] Chlorpyrifos [43]
Touch-induced escape responses (touch response)	22-27 hpf	Touch	DDT [44] Dieldrin [44] Fipronil [44] Nonylphenol [44]
Optokinetic response (OKR)	73-80 hpf	Moving objects	Digoxin [45] Gentamicin [45] Ibuprofen [45] Minoxidil [45] Quinine [45]
Optomotor response	5 dpf	Moving objects	Bisoprolol [46] Chlorpromazine [46]

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			Cisapride [46] Cisplatin [46] Gentamicin [46] Nicotinic acid [46] Quinine [46]
Startle responses	5 dpf onwards	Vibrational or acoustic stimuli	Lead [47] Mercury [48]
Shadow response	8 dpf	Looming shadows	2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) [49]
Prey capture	9 dpf	Prey	2,4-Dichlorophenoxyacetic acid (2,4-D) [50]

4.1.2. Adults

Most assays for locomotor behavior in larvae focus on changes in speed or distance. However, neurotoxic effects can frequently be observed as changes in posture or form of swimming movements [51,52]. Little and Finger demonstrated that the lowest toxicant concentration that produced changes in adult locomotor behavior lies between 0.1 and 5.0 percent of the lethal concentration [51]. However, speed and distance are not the only possible endpoints, nor are they the most sensitive; behavioral endpoints that can be quantified through movement analysis include acceleration, turning angles or frequency, time spent in different swimming modalities (normal swimming, large movement swimming, small movement swimming, burst swimming, etc.), horizontal and vertical distribution of individuals, path tortuosity, and startle responses [52].

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4.2. Anxiety-like behavior

Many options exist currently to assess anxiety-like behavior in fish, most of them using zebrafish [28,53]. The novel tank and light/dark preference tests involve measures of spatio-temporal distribution (time at the bottom of a novel tank or at the dark portion of a light/dark tank) and ethogram (freezing, erratic swimming, risk assessment, thigmotaxis) that are sensitive to anxiolytic or anxiogenic treatments [25]. In a recent meta-analysis, we have shown that the light/dark test is more sensitive to treatments in general than the novel tank test, and that both tests elicit a significant cortisol response equally [25]. It has been observed that standardized behavioral practices within the laboratory such environmental conditions (test days and batches of fish) may have relatively few experimental effects on the outcomes of anxiety and locomotor activity [54]. Examples of toxicants which have been shown to affect anxiety-like behavior in the zebrafish light/dark and novel tank tests can be found on Table 4.

Toxicant	**Concentration / dose range**	**Duration of treatment**	**Test**	**Ref.**
Atrazine	5 - 3,125 µg/L	4 weeks	LDT	[55]
Methylmercury	1 - 5 mg/kg	Acute	LDT, NTT	[56,57]
PCB126	0.3 - 1.2 nM	Developmental (4 - 24 hpf)	NTT	[58]
Dimethyl sulfoxide	0.05%	Acute	NTT	[59]
Copper	0.006 mg/L	Acute	LDT, NTT	[60]

Alterations in anxiety-like behavior are relevant not only to antipredator defenses,

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but also to foraging and resource finding: if an animal is “too cautious” (i.e., increased anxiety-like behavior) due to the effects of a toxicant, it can miss important opportunities to reproduce or to forage outside its home range. Conversely, decreased anxiety-like behavior can lead to “reckless” behavior that ends in being attacked by a predator. The standardization offered by these behavioral assays can help researchers identify alterations in this endpoints, which can lead to novel hypotheses on the ecotoxicological sublethal effects of substances at complex behaviors.

4.3. Aggression

Agonistic and aggressive behavior is associated with territory defense, in agonistic interactions within a social group, in contests for mate access or food, as well as in prey capture and antipredator behavior. In agonistic interactions, fights are usually substituted for ritualized activity (aggressive displays) through which one of the contestants show its superiority without the need to hurt or kill its opponent, or to hurt itself; agonistic interactions can be an appetitive element of aggression in that it can escalate to actual aggressive behavior, or towards a resolution [61].

Aggressive-like behavior has been studied in laboratory fish with different approaches, ranging from mirror tests (in which an aggressive display are elicited by mirror images) to dyadic fights and group social interaction [29]. The advantage of using the mirror test is that it can capture most elements of aggressive motivation without unnecessarily risking damage to the animals; however, since no resolution is possible in mirror-elicited displays, the full range of behaviors and physiological adjustments is not

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captured. Choosing between these alternatives involves balancing ecological validity, throughput, and welfare concerns.

Alterations in aggressive behavior can potentially decrease fitness by increasing the likelihood of losing a contest, getting damaged after inadequately escalating the fight, or losing access to resources such as territories or food. Table 5 represents some examples of the effects of toxicants in aggressive and agonistic behavior in zebrafish.

Toxicant	**Concentration / dose range**	**Duration of treatment**	**Test**	**Ref.**
17α-ethinylestradiol	0.5 - 50 ng / L	48 h	Dyadic interaction	[62]
	0.4 - 2.2 ng / L	14 days	Group interaction	[63]
TBBPA	5 - 50 nM	Developmental (1 - 120 dpf)	Mirror test	[64]
Methylmercury	1 - 15 µg / L	32 h	Mirror test	[65]
Paraquat	20 mg/kg	6 injections for 16 days	Mirror test	[66]

5. Moving from behavioral toxicology to ecotoxicology: Ecologically-relevant endpoints

The tests and behavioral bioassays that were reviewed in Section 3 are all sensitive to subtoxic concentrations of important environmental contaminants. They also

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have the advantage of being easy to implement in carefully controlled laboratory environments; differently from behavior observed in the field, variables in the laboratory can be cautiously manipulated to produce the most reliable and sensitive measurements that can satisfy regulatory agencies. However, it is not always clear how specific endpoints (e.g., caudal fin tremors [67]) are related to responses at the individual and higher levels which are of interest to ecotoxicology

Almost 35 years ago, Rand [68] suggested that the behavioral responses which are more useful for toxicology include those that are (A) well-defined and practical to use; (B) sensitive to a range of contaminants and observable in different species; (C) with known environmental factors; and (D) ecologically relevant. Among these criteria, the first three are amenable to laboratory testing, while the last is usually hypothetical. Increasing ecological relevance can be reached by at least two approaches: the use of neurobehavioral domains, which imply in running more behavioral tests; and analyzing the relationship between behavior in the laboratory and behavior in the wild.

The concept of a behavioral domain is widely recognized in behavioral genetics of knockout and mutant laboratory animals [69], in which recognizing whether the effect of a given genetic manipulation is specific to the test or generalizes to a more general domain is important. Behavioral domains of interest to neuroscientists (and, as an extension, to neurotoxicologists) include anxiety, mood, social behavior, cognition, and impulse control. Many of these domains are, in hypothesis, related to the complex behaviors observed at the individual level that are shown in Figure 1. Thus, well-controlled laboratory experiments, using more than one test, can determine whether the effect of a given toxicant impacts one or more behavioral domains which are likely to

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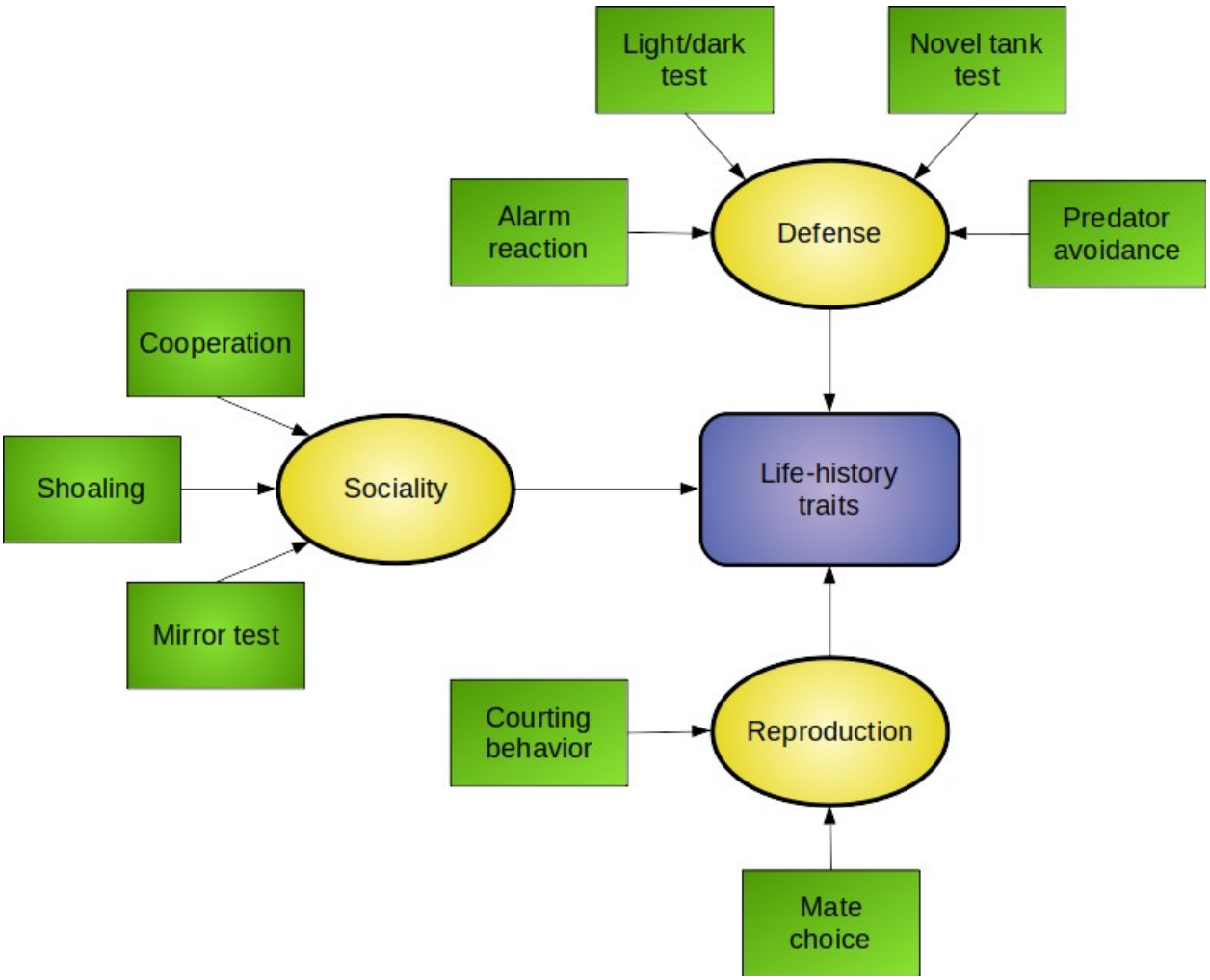
affect these complex responses (Figure 2).



This approach can generate powerful hypotheses that can be further tested in field experiments by toxicologists and ethologists alike, either by direct observation in the wild or by “laboratory in the field” approaches. For example, following observation of natural antipredator behavior in the wild, animals can be captured, taken to the laboratory, and its behavior in standardized assays can be tested to check whether it predicts performance in the wild. Although powerful, this approach is unlikely to be of direct interest of neurotoxicologists.

In summary, the power of behavioral experiments in the laboratory can be tapped

to expand the reach of ecotoxicological approaches by increasing the number of tests with a domain-based mindset. In addition to increasing throughput, this approach is also compliant with the philosophy of the 3R's (reduce, refine, replace) in animal research.

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Figure captions

Figure 1 - Toxicants can produce effects at individual-level responses, which in its turn impact responses at the population and higher (species, community, ecosystem, and evolutionary) levels. Complex behaviors such as reproduction, defense, and sociality can impact life-history traits.

Figure 2 – Well-controlled laboratory experiments, using more than one test, can determine whether the effect of a given toxicant impacts one or more behavioral domains which are likely to affect complex responses (reproduction, defense, sociality) that are related to life-history traits, and likely to impact responses at higher levels.