REVIEWS

Studying behavioural variation in salmonids from an ecological perspective: observations questions methodological considerations

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Abstract Salmonid fish are an ecologically important and extensively studied group of fish which concern many interest groups in our society. The aim of this paper is to discuss and suggest solutions to the multifaceted problems associated with studying behavioural variation in salmonids, with focus on designing behavioural studies that are ecologically relevant. Many of the general problems and solutions discussed can be applied to other animals as well. First, the importance of asking clear questions when conceiving behavioural studies is addressed, using Tinbergen's four questions and associated theories as stepping stones towards generating testable hypotheses about behavioural variation. We then address a range of methodological challenges encountered when

attempting to study behavioural variation in salmonids and suggest solutions to overcome these problems. A range of approaches is discussed, from highly controllable laboratory experiments to monitoring studies of behaviour in the wild. The importance of combining lab- and field approaches to evaluate the ecological relevance of behavioural variation is highlighted. Finally, we suggest a general framework using a multi-faceted research approach to address questions about the behavioural ecology of salmonids (and other animals) so that knowledge can progress, and the ecological relevance of behavioural studies can be validated.

Keywords Animal personality · Behavioural ecology · Experimental methodology · Multi-faceted approach · Salmonidae

This paper is dedicated to the memory of the ethologist and salmonid biologist Torbjörn Järvi.

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Background: why this paper was written and what it is about

In recent years, there has been an increasing interest for studying individual behavioural variation in animals (Réale et al. 2007; Bell et al. 2009) where fish, including salmonids, have been among the most studied taxa (Conrad et al. 2011; Mittelbach et al. 2014). To understand how behavioural variation is generated and maintained in natural populations is not

only of basic ecological and evolutionary interest (Dingemanse and Réale 2005; Réale et al. 2010; Sih et al. 2012; Mittelbach et al. 2014; Montiglio et al. 2018), but such knowledge is also critical to guide sustainable management and conservation of natural resources (Shumway 1999), for example by better understanding the selective effects of commercial (Killen et al. 2015) and recreational fishing (Arlinghaus and Cooke 2005), and the causes and consequences of biological invasions (Juetten et al. 2014).

Salmonids are among the most important and influential native fish species in the northern hemisphere (Crawford and Muir 2008). They are targets for recreational and commercial fishing, important for aquaculture, part of the cultural heritage of many nations (Gresswell and Liss 1995; Newton 2013), and provide substantial marine-derived nutrient deposits to river ecosystems (Helfield and Naiman 2006). Moreover, because of their value, salmonids have been translocated widely outside their natural range since the late nineteenth century (Newton 2013; Hutchings 2014). Five species, brown trout *Salmo trutta*, Atlantic salmon *Salmo salar*, rainbow trout *Oncorhynchus mykiss*, brook charr *Salvelinus fontinalis* and lake trout *Salvelinus namaycush*, are listed in the IUCN Global Invasive Species Database (Pagad et al. 2015).

Despite rarely being recognized among the classical model organisms of biology, several of the salmonid species are extensively studied (Elliott 1994; Jonsson and Jonsson 2011; Hutchings 2014), and the results are of interest not only to biologists, but also to many other interest groups in our society (e.g. commercial and non-commercial fishermen [including a number of indigenous peoples], anglers, fisheries managers, nature- and cultural conservation agencies, hydroelectric companies, river trusts, aquaculturists, fish consumers, and conservation NGOs). It is likely fair to say that few other wild species have a wider range of interest groups being concerned about them, in one way or another, than the species of the salmonid family [especially the salmonine subfamily, which includes trouts, salmon, and charrs (Horreo 2017)]. Consequently, there is an impressive amount of information available about their biology and ecology, which makes this group of fish suitable as models for studying behavioural variation from an ecological view.

The aim of this paper is to discuss and suggest solutions for how to deal with the many challenges

associated with studying behavioural variation in salmonids, particularly the challenge of designing behavioural studies that are ecologically relevant. This perspective is chosen because although we agree that “nothing in biology makes sense except in the light of evolution” (Dobzhansky 1973), *the ecological reality that interplays with evolution is what all organisms have to cope with*. Although salmonid fish will be used as model organisms, the questions and problems are general and can be applied to studies of other species as well. Observation is the starting point from which questions about behaviour can be addressed at different levels. The classic framework of Tinbergen’s four questions (Tinbergen 1963) will be used to highlight the importance of being clear about asking questions when conceiving a behavioural study. The core of the paper addresses a range of methodological challenges encountered when attempting to study and measure behavioural variation in salmonids, followed by suggestions of solutions to overcome these problems. Finally, based on our own and other researchers’ experiences from conducting and combining lab- and field studies on salmonids, we suggest a general framework for how to approach questions about the behavioural ecology of salmonids (and other animals), and how the ecological relevance of behavioural studies can be validated, using a multifaceted research approach. We do not discuss statistical methods for analysing behavioural variation. This important aspect is treated in other, topical publications that are highly recommended (e.g. Nakagawa and Schielzeth 2010; Dingemanse and Dochtermann 2013; Westneat et al. 2015; Allee et al. 2017; Wilson 2018). Furthermore, while the overall focus relates to the importance of behavioural variation in natural salmonid populations, many examples stem from research on salmonid behaviour more generally, both in nature and in standardized experimental environments. Thus, we build our arguments from the wealth of the fish literature in general and the salmonid literature in particular, suggesting ways forward to extend the research community’s already extensive knowledge about these fascinating animals.

Why all this variation?

Along with many colleagues, we have typically worked with a combination of lab- and field studies,

often using brown trout, but also other salmonids, as models. Often, juvenile trout are captured in streams by electrofishing and brought into the lab for behavioural observations and experiments. If these young trout are placed in small aquaria and introduced to a novel object, they will show a tremendous variation in their behavioural response, which is striking to the observer (Fig. 1). Some individuals appear to inspect the object intensively and closely whereas others seem to “freeze”, not moving at all. Other responses include swimming stereotypically along the side of the tank, as if the fish is stressed and attempts to escape. It is easy to fall into the trap of anthropocentric interpretations of personalities when these observations are filtered through the cognitive biases of humans (Epley et al. 2007). But what is it, really, we are observing? Small juveniles have a high metabolic rate and grow faster than larger individuals (Bohlin et al. 1994; Elliott and Hurley 1995). Maybe their individual behaviour reflects differences in foraging motivation triggered by variation in energetic status, i.e. hunger levels (Johnsson et al. 1996)? Maybe environmental factors, such as light conditions (Varanelli and McCleave 1974; Enefalk et al. 2017), water temperatures (Gibson 2015; Forsatkar et al. 2016), and/or electromagnetic fields (Varanelli and McCleave 1974; Lee et al. 2015), in the lab vary

spatially and temporally, generating the behavioural variation? Or are we observing different consistent personalities among individuals as resulting from genetic (Bakker 1986), parental (Burton et al. 2016), epigenetic (Ledón-Rettig et al. 2013), or environmental (Sundström et al. 2003) effects? In fact, there may be interactions among several causal factors, e.g. between food availability during ontogeny and maternal effects (Jonsson and Jonsson 2014; van Leeuwen et al. 2016). To find out if behaviour is consistently different among individuals, observations need to be repeated on the same individual. However, as an experiment progresses in time, the individuals may habituate to the situation (Finger et al. 2016), which in turn may alter their behaviour and make them either converge or diverge in their performance, making repeatability estimates hard to interpret (Killen et al. 2013; Biro and Stamps 2015)? And finally, how do we know that this lab-scored behaviour reflects behaviour under natural conditions, and hence is relevant to understanding ecology and evolution (Adriaenssens and Johnsson 2009; Bell et al. 2009; Killen et al. 2013)? Armed with these challenging but inspiring questions we will now move to the next chapter: asking questions about behaviour, using a helpful framework presented by Tinbergen (1963).

Fig. 1 Movement tracks for 14 juvenile brown trout individuals during a novel-object test. Modified from Adriaenssens (2010)

Tinbergen's four questions about behaviour

When researchers are asked about which specific research question or hypothesis they are addressing in a behavioural study, they are surprisingly often not able to give a clear answer, and it has recently been made clear that sometimes the questions and predictions are conceived ad hoc in the field of ecology and evolution (Fraser et al. 2018). While exploratory research has its value in generating novel hypotheses (e.g. Tinbergen 1963), clearly formulated questions and hypotheses are critical components for aiding interpretation of experimental results (Mayr 1961) and are also important for reducing the temptation to invent ad hoc explanations for surprising results (Kerr 1998; Fraser et al. 2018). As shown in the previous section, it is possible to generate many explanations for the behavioural variation illustrated in Fig. 1. Fascinating as it is, mere observation and description is in the end insufficient to explain and rigorously understand animal behaviour—it is just a starting point.

In a seminal paper in the advent of ethology, Nikolaas Tinbergen formulated a four-pronged framework of questions for a more complete understanding of animal behaviour (Tinbergen 1963). These questions relate to (1) the direct physiological causation underlying the expression of the behaviour (the *mechanism*), (2) the function of the behaviour in increasing the fitness of the animal (the *adaptation*), (3) the development of the behaviour through the life of the animal (the *ontogeny*), and (4) the facilitation and constraints posed on the possible behavioural expression by the evolutionary history of the species (the *phylogeny*). Despite being conceived more than half a century ago, Tinbergen's multi-level approach is still the strongest framework for conceiving and designing behavioural studies, and his structured approach is still highly acknowledged and discussed (MacDougall-Shackleton 2011; Bateson and Laland 2013). In the following section we will apply Tinbergen's four questions to one observation of salmonid behaviour, the seaward migration performed by Atlantic salmon (Fig. 2).

Young salmon migrate to sea—how and why?

Observing a shoal of 2–4 year old Atlantic salmon migrating toward the sea in spring will lead a student

of salmonids to ask questions about why they do so. A first answer could be given at the level of physiological causation:

Since increasing day-length and temperature trigger a suite of physiological changes, which in turn alter the ontogeny and the behaviour of the young salmon (a phenomenon known as smoltification), it will shift from maintaining a position against the current in the stream and start moving downstream (Björnsson and Bradley 2007).

This answer does, however, not provide information about why this would happen at this particular time in life (why not earlier/later?), so an additional answer will have to elaborate on this:

Because the salmon needs to reach a certain size (ontogenetic stage) to be able to survive the migration, it will have to wait until it is large enough; depending on the growth capacity and -opportunities the actual age-at-migration will differ among individuals and across geographical areas (Metcalf et al. 1988).

So, now we have made clear that the migration also depends on ontogenetic constraints. We have still not explained why this particular behaviour would be favoured at all, especially since more potential predators are present in the sea (Hvidsten and Lund 1988), so a third level of explanation is required for this question:

Seaward migration in salmonids is an adaptation that has evolved to take advantage of the richer food sources and larger feeding areas in the sea (McDowall 2001). Thus, the migrating salmon reach much larger sizes than the freshwater resident conspecifics and achieve a higher reproductive success by increased fecundity (in females) and superior ability to compete for females (in males) (Fleming 1996); such fitness benefits can outweigh the increased risk of mortality associated with seaward migration.

With this answer, we have pinned down a rough overview of the proximate and ultimate reasons for why the Atlantic salmon juvenile show the migratory behaviour. However, a persistent student will still wonder why not all stream living fish do this, if it is so beneficial. To answer this, we need to visit the final

Fig. 2 Tinbergen's four questions (Tinbergen 1963) applied to the multiple levels of explanation for why young salmon migrate to the sea. The dichotomy of proximate and ultimate explanations is adopted from Mayr (1961)

stage in Tinbergen's framework, the phylogenetic explanation:

Species differ in their capacity for undertaking diadromous migrations. The ancestors to different species evolved different strategies, and those evolutionary patterns can still be retained in current-day species. The exact pattern of the evolution of migration in salmonids may not yet be completely resolved, but Atlantic salmon share the migratory behaviour with several closely related species, suggesting that the predisposition for migration originated before Atlantic salmon became a distinct species (McDowall 2002; Alexandrou et al. 2013; Horreo 2017). Hence, the Atlantic salmon, and many of its nearest relatives, can utilize an adaptation evolved in their common ancestor; something that is not available to some other stream-living species, simply because their ancestors did not evolve some key traits (e.g. salinity tolerance). That doesn't mean that other species are inferior, they only ended up on a different path to fitness.

With this fourth answer, we have responded to all four of Tinbergen's questions. These multiple levels of explanation, which some researchers like to divide into proximate (mechanistic, ontogenetic) and ultimate (adaptive, phylogenetic) explanations (cf. Mayr 1961) are non-independent from each other, and many more questions can be posed to dig deeper into the

details of salmon migration. However, the main point to be made is that answering all these questions, in an integrated way, is required for the deep understanding of the behaviour in question (MacDougall-Shackleton 2011; Bateson and Laland 2013). With respect to integrating the explanations, much is still left to do in ethology. The complexity of genetic, parental, and epigenetic inheritance that may cause transgenerational effects linking proximate and ultimate levels of explanation in new ways and blur their distinction (Laland et al. 2011). Yet, the core of Tinbergen's framework is still standing and is very useful to identify and address specific question(s) when studying variation in behaviour (and other phenotypic traits) at the individual-, population-, and species level.

Tinbergen's questions are a first guiding step to generate questions and hypotheses explaining variation in behaviour. We should also integrate relevant ecological and evolutionary theories, including e.g. the theories of frequency-dependent selection (Maynard Smith 1974), parental bet-hedging (Cooper and Kaplan 1982), fluctuating selection (Dingemanse et al. 2004), growth-mortality trade-offs (Stamps 2007) and the pace-of-life syndrome hypothesis (Réale et al. 2010). An example addressing the salmon smolt-migration discussed above concern the theory of frequency-dependent selection which has been applied to explain why some males refrain from migrating to the sea and mature precociously to instead adopt a sneaky reproductive tactic (see e.g. Gross 1985).

Methodological considerations

In this section, some frequently encountered challenges and key problems associated with studying and interpreting individual behavioural variation in the lab will be discussed. These are problems that our research group have encountered over the years, as well as experiences from interacting with other researchers in the field. We start with discussing general aspects like the importance of acclimatisation, sampling procedures, and which fish to use moving on to the challenges associated with measuring specific behaviours in the lab. While we do not delve into it here, it is worth pointing out that combinations of different challenges and problems may also interact to produce other issues than the ones presented.

Acclimation to the test environment

The procedure of acclimating salmonids to lab conditions for behavioural studies is strongly dependent on fish age and size, temperature, light conditions and many other factors, as well as the specific aims of the study. Some studies indicate that salmonids may require up to a week to fully recover physiologically from the stress effects of handling during capture (Ejike and Schreck 1980; Barton and Schreck 1988), but it is still difficult to provide general advice and sometimes pilot testing is needed, especially if acclimation times must be minimized due to experimental time constraints. Furthermore, a drastic change in the physico-chemical environment (salinity, light-cycle, temperature, etc.) typically enforce unavoidable physiological changes and require some additional time for acclimatisation before the physiological functions return to normal and this will also impact on behavioural performance. For instance, for a smoltifying Atlantic salmon the behavioural performance in saltwater (e.g. the anti-predation response) will depend on the degree to which it has fulfilled the smoltification (Järvi 1989).

In general, smaller/younger salmonids acclimate faster to laboratory conditions than larger/older ones. There are likely several non-exclusive reasons for this. One is that smaller individuals have a higher metabolic rate, it is adaptive to grow fast since a small body size makes fish vulnerable both to starvation and to predation from a larger range of gape-limited predators (Godin 1997; Näslund and Johnsson 2016b). For

wild salmonid fry, it generally takes less than 24 h to initiate feeding in captivity. As the fish grow larger and older, acclimation times generally increase; in 1 year-old parr, feeding in the lab may commence first after a week of captivity. This is likely due to a combination of factors which may include ontogenetic changes affecting the growth-mortality trade-off (Lima and Dill 1990). For instance, larger individuals generally have larger energy reserves and therefore energetically can afford to postpone feeding, to the benefit of vigilance, when perceiving a threat. Older salmonids can also become more crepuscular/nocturnal than fry (Gries et al. 1997). However, circadian activity patterns differ among individuals (Richardson and McCleave 1974; Alanärä et al. 2001; Závorka et al. 2016), and this could potentially lead to different acclimation times for different individuals. Presenting the fish with natural food rather than pellets, especially living food that can survive a long time in water (like earthworms), can reduce the time it takes for the fish to start to feed. Acclimation times can also be affected by the development of cognitive ability as the fish grow older (Petrazzini et al. 2014). This is indirectly supported by the fact that mirror image stimulation aggression tests work well on young-of-the-year brown trout, but are more difficult to carry out on older brown trout (Bohlin et al. 2002a) as they may have a more developed cognitive machinery to detect the “odd behaviour” of the mirror image (Desjardins and Fernald 2010). Acclimation can also be affected by problems associated with scaling up experimental facilities, aquaria etc. to the size of the fish. In particular, this can be a problem for studying larger salmonids which require sufficient space to perform natural behaviour (Jenkins 1969; Fausch 1984), for example when performing spawning studies on sea-run salmon which require large and advanced experimental set-ups (for good solutions, see Fleming and Gross 1993; Fleming et al. 1996; Petersson et al. 1999).

The problem of acclimation could possibly be turned to the researcher’s advantage in the study of behavioural variation. Individual variation in acclimation time has, for instance, been linked to boldness in pumpkinseed sunfish *Lepomis gibbosus* (Wilson et al. 1993). In principle, this could be a valid boldness assay for salmonids as well. However, such an approach requires that the field capture method does

not induce variation in stress levels among captured individuals, since this may lead to bias in the scoring.

Effects of the physical and social environment

When studying wild fish behaviour from an ecological perspective, you would presumably want their behaviour in the lab to resemble their behaviour in the wild and also, from an evolutionary perspective, how this behaviour may affect the probability of the individuals' genes being represented in the next generation. Although ecological and evolutionary relevance is often assumed in lab studies of behaviour, the extrapolation is far from straightforward. Many fish species, not least salmonids, are highly plastic in their behaviour, and alterations of the captive environment, as well as its similarities with the natural environment that the fish evolved in, can have dramatic effects on expression of individual behaviour (Killen et al. 2013; Johnsson et al. 2014). Still, phenotypic plasticity is limited, and the performance of different phenotypes depends on the environment. For instance, studies suggest that Atlantic salmon individuals that perform well in captivity tend to perform poorly in the wild and vice versa (Saikkonen et al. 2011). For example, growth in the wild can be affected by different genes than growth in the captive environment (Vasemägi et al. 2016). Thus, the fitness effect of a certain behavioural phenotype is often environment-specific and especially sensitive to evolutionary novel environments, like laboratory aquaria and hatchery tanks (Ghalambor et al. 2007). Consequently, it is important that the conditions in the lab mimic natural conditions as much as possible. However, this often involves trade-offs between realism and efficiency and pilot studies on a small subsample of fish is often helpful to find the best compromises before initiating the main experiment. For example, many stream-living salmonids avoid strong light, because it makes them more conspicuous to potential predators (Valdimarsson et al. 1997), and show less stress under dim light conditions. The trade-off here is to still have enough light for the video software (or your own eyes when recording manually) to be able to distinguish the fish against its background. Similarly, most stream-living salmonids prefer (Johnsson et al. 2000) and benefit from physical structure in the tank which can fill several purposes. Salmonids typically maintain contact with the substrate in their resting position

(Kalleberg 1958), and this can be especially critical for yolk-sac fry, which risk deformation of the yolk sac and waste a lot of energy through unnecessary activity when no substrate is present (reviewed in Näslund and Johnsson 2016a). Rocks and plants can also facilitate feeding and reduce stress in fish, both reared individually and in groups. For example, brown trout has been shown to prefer over-head cover over open areas in tanks, likely because it is perceived to provide visual protection from terrestrial predators (Johnsson et al. 2004). In groups, physical structures reduce the number of fin injuries (Näslund et al. 2013), likely due to reducing visual contact (Kalleberg 1958) and by providing refuges for subordinate individuals from aggression by dominants (see e.g. Jenkins 1969), and can even affect the relative growth and survival of dominant and subordinate fry (Höjesjö et al. 2004). Thus, physical structure can be used to meet life-stage specific requirements and to provide a more nature-like environment, but compromises are required for behavioural studies where individuals need to be constantly visible for behavioural scoring. For a more comprehensive review of the effects of physical structure on captive salmonids and for fish rearing in general, see Näslund and Johnsson (2016a).

The size of the trial arena is another obvious feature that needs to be considered in all studies, since the arena size in relation the fish size likely affects basic behavioural variables such as movement activity (Polverino et al. 2016). When using arenas consisting of several compartments, the size of openings between compartments may also affect the recorded expression of behaviour, and this effect itself may depend on other aspects of the arena design, such as environmental complexity within the arena, as well as the population-origin of the investigated fish (Näslund et al. 2015).

Features of the physical and social environment are interdependent and share some basic characteristics. For example, a shoal of pelagic fish provides both a social and physical environment to the individual (Krause and Ruxton 2002; Katz et al. 2011). Thus, the physical pre-conditions for social interactions need to be considered with respect to the aim of the study and the species studied. Species that are territorial, like brown trout, are more aggressive than group-living species, and often perform well when alone in the tank. In contrast, salmonids tending to associate in groups in nature, like Arctic charr *Salvelinus alpinus* (Jansen

et al. 2002) may need the company of conspecifics to perform adequately, although growth and shoaling behaviour increases, while aggression decreases in larger groups (300 ind.), as compared to medium (150 ind.) and small groups (75 ind.) (Brown et al. 1992). In Arctic charr, the benefits of sociality also seem to depend on the phenotype, as larger individuals perform better in small groups (6 ind.) than smaller fish do (Leblanc et al. 2011). All the factors discussed above (light, physical structure, tank size, etc.) will potentially affect the outcome when studying social behaviour, but the optimal design is dependent on the specific research question asked, as well as on the species investigated. General knowledge about the model species is imperative when designing all kinds of laboratory studies, but when it comes to sociality, it is critically important. Experimental setups where fish are maintained at higher-than-natural densities may benefit from including a natural-density control treatment, if the aim is to predict natural behaviour.

Rearing density is a key factor influencing social interactions in salmonids in multiple ways, in addition to the crowding stress responses associated very high densities, e.g. in food fish farming (Johnsson et al. 2014).

Firstly, as the cost of territorial defence increases with competitor density (Grant 1997), increasing the number of competitors tend to reduce the pay-off of territorial defence and aggression in brown trout (Kaspersson et al. 2010) and rainbow trout (Pettersson et al. 1996). Still, aggression can be high in small groups (3–10 individuals) which to some extent is a consequence of the closed laboratory environment, i.e. subordinate individuals cannot move out of sight of the aggressor (Kalleberg 1958). These problems can at least partially be overcome by introducing physical structure, as mentioned above (Höjesjö et al. 2004). However, aggression may also occur at high densities as fin nipping may increase due to stress and/or induction of scramble competition for food (Cañon Jones et al. 2011). Thus, the mode of food delivery is also very important, as the spatial and temporal distribution of food affects aggression and growth variation (Grant 1997).

Secondly, many salmonid species can form social hierarchical groups where aggression decreases with time due to familiarity as social ranks become stabilized. Thus, reshuffling of groups of fish may result in increased aggression which can also be used

deliberately to study the effects of familiarity on behaviour (Griffiths et al. 2004; Závorka et al. 2015). However, with increased group size, the cognitive demands associated with recognizing individuals are expected to increase so familiarity and associated behavioural changes may not be realized, as predicted by the hypothesis of limited attention (Dukas and Kamil 2001).

Thirdly, increasing the number of individuals (i.e. density) in a group may reduce the efficiency of individual learning, as the visual field and activity space of individuals may be restricted and, depending on other aspects of the experimental design, individuals may therefore switch from individual learning to using social information and copying the behaviour of others (Brown and Laland 2003; Kendal et al. 2004; Brockmark et al. 2010). Correspondingly, several studies suggest that rearing density has strong effects on the development of adaptive individual behaviour in young brown trout where (unnaturally) high rearing densities impair adaptive behaviour in the lab, in turn resulting in reduced survival and growth upon release in nature (Brockmark and Johnsson 2010; Brockmark et al. 2010). In general, these studies illustrate the advantage of combined approaches that allow behavioural variation in the lab to be translated into fitness-related traits in the wild. Combined approaches will be considered more in depth later on.

It is also important to consider relatedness among individuals in studies involving social behaviours, as close kin may be expected to be less aggressive and competitive towards each other than unrelated individuals, due to kin selection (Griffiths and Armstrong 2002).

Unrepresentative sampling

Since individuals often differ in aggression, boldness and curiosity (Bell et al. 2009), researchers studying individual variation need to be careful when sampling because they may easily end up with a biased sample not representing the full range of behavioural variation in the population (Biro and Dingemanse 2009). In addition, this problem may be accentuated by the fact that behavioural characteristics often are correlated with physiological traits like swimming capacity and agility (Killen et al. 2016) which may further bias sampling as fast swimmers are more likely to escape. However, the direction of such effects is not always

intuitive and strongly dependent on the method of capture. Active trawling is hypothesized to selectively remove shy and reactive behavioural phenotypes (Killen et al. 2015). In contrast, passive fishing methods like baited traps, netting, and angling (Hubert et al. 2012) may selectively catch individuals with bold-like behaviour (i.e. explorative, active, aggressive, and risk-taking phenotypes) (Härkönen et al. 2014; Lennox et al. 2017). Bold-like behaviours may also be linked with diurnal activity patterns (Závorka et al. 2016), making time-of-day a potential factor to consider when conducting representative sampling. Under some conditions, field sampling may also result in genetic bias. When sampling young fish in a natural stream, there may be an increased probability of catching siblings close to each other and, since many traits including some behavioural traits and their covariance (Kortet et al. 2014), are heritable, sampling from a single stream site may result in reduced variation in trait expression that is unrepresentative of the population. However, this may not be a major problem if the population is large, with many parental fish spawning in the same area (Fontaine and Dodson 1999). There is no easy solution to the problem of biased sampling of free-living fish. It is important to be aware of the problems and experienced with alternative capture methods and their efficiencies, so the method of least bias can be chosen. Furthermore, sequential removal of individuals from their habitat (e.g. using multi-pass electrofishing) can give information about the direction of the bias of a certain sampling method, aiding in the evaluation of whether the sampling has been representative as well as in the interpretation of the results (Adriaenssens and Johnsson 2013).

In hatcheries or laboratories representative sampling can generally be achieved relatively easily by being cautious when sampling and distributing fish from holding tanks. To resolve this issue, it is advisable to anaesthetize fish prior to sampling and use predetermined treatment allocation, or allocation concealment procedures, to reduce selection bias during sampling (e.g. Ruxton 2017). It is also possible to reduce water levels in tanks to equalize catch probability among individuals. However, this type of haphazard assignment could still benefit from being followed by sedation and measurements of e.g. length or mass, to check whether the sampled groups at least show similar distributions in these traits. Also,

handling and prolonged and/or substantial reduction of water levels will likely lead to stress reactions which in turn may increase acclimatization time and affect subsequent behavioural performance and general welfare (Barton et al. 1980; Einarsdóttir and Nilssen 1996; Huntingford et al. 2006). Stress may be particularly important to consider when specifically investigating different behavioural types (i.e. personalities) in the lab (Killen et al. 2013). On the one hand, different behavioural types are generally assumed to be associated with stress coping styles (Koolhaas et al. 1999; Øverli et al. 2007), and if this is the case, subsequent behavioural scores may become biased. On the other hand, it is possible that only a stressful situation can induce the behavioural differences that the researcher is interested in, making stress a vital part of the experimental design. Context dependence of behavioural variation and its consistency, in relation to any environmental factor, is likely very common (Killen et al. 2016), and therefore requires attention when designing experiments.

Bias in the motivation to perform

When conducting behavioural trials, it is important to standardize the motivational state of the subject individuals. Hunger is a factor affecting motivation which in turn potentially affects behavioural scores in standardized trials (Symons 1968; Höjesjö et al. 1999; Vehanen 2003) and in dominance studies (Johnsson et al. 1996). Most studies aim to standardize hunger by either feeding all fish *ad libitum* or starving for a specific time prior to trials. Out of these two options, starvation seem to be the most reliable standardization, as (1) individually reared fish commonly differ in their food intake in relation to their size, and (2) the food distribution among individuals in salmonid groups is often unevenly distributed as a consequence of dominance hierarchies, with subordinates being suppressed even if food is given in excess (Abbott and Dill 1989; Huntingford et al. 1993). However, even if food intake is perfectly standardized, some issues will remain. For instance, when aiming to investigate cognitive capacities in different groups of salmonids, the motivation to solve a cognitively demanding task may differ among the groups, and results will thereby be confounded. Hatchery fish are raised in an environment lacking natural predators and are therefore commonly more motivated to feed, and thereby

have higher activity than wild fish. In a cryptic prey search, or in a maze trial, higher activity levels will result in the hatchery fish solving the task faster than a more cautious (and less motivated) wild individual (Adriaenssens and Johnsson 2011). At the same time, the hatchery fish tend to make more errors, so while solving the task quicker, the cognitive function appears to be impaired in hatchery fish (Adriaenssens and Johnsson 2011). The severity of motivation issues likely varies depending on life stage. For instance, recent food restriction of salmonid fry appears to have only minor, if any, effects on behaviour in standardized trials, likely a consequence of a constant high motivation to forage, driven by the necessity to outgrow the vulnerable fry stage (Näslund and Johnsson 2016b; Näslund et al. 2017a,b). In contrast, at the parr stage hunger effects tend to increase activity, boldness and aggression (e.g. Symons 1968; Höjesjö et al. 1999; Vehanen 2003).

Hormone manipulations, as mediated by either implants or transgenesis, typically influence motivation, which should be kept in mind when comparing behaviour of hormone manipulated individuals with controls. For instance, GH implanted rainbow trout receive higher aggression scores due to higher activity resulting in higher encounter rates with opponents (likely as a consequence of higher feeding motivation), than control individuals (Jönsson et al. 1998). Despite higher aggression, these fish are not more dominant. Similar effects can be seen in brown trout implanted with ghrelin, a GH stimulating hormone (Tinoco et al. 2014). Similarly, hatchery reared brown trout invest more time and energy in territorial conflicts than wild fish without increasing their probability of dominance (Deverill et al. 1999; Sundström et al. 2003). Increased feeding motivation also increases individual food intake in GH implanted rainbow trout (Johnsson and Björnsson 1994), and GH transgenic coho salmon (Devlin et al. 1999), and also likely contributes to the reduced anti-predator behaviour in GH transgenic Atlantic salmon (Abrahams and Sutterlin 1999).

The smoltification process also affects motivation to perform certain behaviours. In shelter-seeking trials, for instance, testing fish at the pre-smolt stage is largely futile, as these fish have changed their motivation to seek shelter in the complex river bottom structure into a strong motivation for migrating seawards (see Fig. 2; Rosengren et al. 2017), albeit

apparently with some conflicting “travelling anxiety” (see Hellström et al. 2016).

Risk perception and the interpretation of boldness studies

Boldness is a commonly measured trait in fish studies, although the definition of what exactly constitutes boldness is somewhat obscure, since different boldness traits do not always correlate within individuals (e.g. Burns 2008; Toms et al. 2010; Ólafsdóttir and Magellan 2016). In general, boldness is scored in a situation where the animal is confronted with a potentially risky situation. Depending on the decision made in the situation the individual obtains a score on the bold-shy continuum. Examples of tests aimed at scoring boldness-like behaviours are open-field tests (where bolder fish are assumed to spend more time in the centre of an open arena), shelter emergence tests (where bolder fish should have shorter time-to-leave), and novel-object tests (where bolder fish have more interactions or shorter distance to a novel object; see Fig. 1). While these tests have face validity (i.e. they are intuitive; Carter et al. 2013), it remains difficult to prove that they are accurate determinants of boldness. For instance, if we assume that all fish have equal boldness, but differ in activity, the more active fish might very well approach a novel object more often. Another problem arises if the object is not considered as a potential threat to the fish, which would be indicated by randomly distributed distances to the object over time (see e.g. Näslund and Johnsson 2016a). In the same vein, correlating boldness with other traits, such as e.g. foraging activity, may not lead to any viable results if the environment in which the additional trait is scored is not considered risky. For instance, swimming in an open tank may not be considered particularly risky for fish that are acclimated to a predator-free laboratory environment, and hence it would make little sense for a shy fish to be less active than a bold fish in this situation. Boldness-measures may also be affected by ontogenetic changes in behaviour (as is the case in the early life of brown trout; Näslund et al. 2015), making interpretations difficult if not all examined fish are within the same stage of life. In general, studies targeting boldness would benefit from validation in real risky situations. However, this is often associated with ethical considerations, since real risky situations are not benign to

the fish. A second-best alternative may be to conduct more than one test of boldness. If the test-scores covary, then it may be appropriate to term the composite score ‘boldness’; if not, then it might be better to give the separate scores more detailed labels.

Restricted movements in dominance and competition studies

Allowing fish to leave an experimental area is a critical, yet hard-to-design, feature for the ecological relevance of dominance studies. Both laboratory and small sized mesocosm experiments are difficult to conduct while maintaining an ‘open’ environment where individuals can come and go as they wish. For instance, results showing that the individual ranked second best in competitive ability is the one that suffers the most within a dominance hierarchy (e.g. Sloman et al. 2000; Závorka et al. 2015), may be experimental artefacts caused by restrictions in the space in which the fish can move (Sloman and Armstrong 2002). In the wild, the second ranked individual would likely try to find another place to dominate, instead of sticking around a superior competitor and get consistently beaten up. The result from the lab gives important insights into the dominance hierarchy (i.e. that the strongest individual mainly focusses on attacking the most competitive intruder, while accepting the presence of less competitive individuals), but extrapolation to natural conditions should be made with caution. In brown trout, for example, parr that are non-aggressive in dyadic contests in the lab can grow as fast in the wild as individuals that are scored as aggressive and dominant in the lab (Höjesjö et al. 2002). The serial removal method, i.e. removing the most dominant individual after each observation round until only one individual remains, is a useful method to evaluate the stability and linearity of dominance hierarchies that works well for salmonid fish. However, the method can be time consuming when many and/or large groups need to be studied. Empirical studies using the removal method suggest that established salmonid dominance hierarchies are linear and relatively stable over time in Atlantic salmon (Metcalf et al. 1989), rainbow trout (Johnsson 1993), and brown trout (Adriaenssens and Johnsson 2011).

Large laboratory setups can likely give very interesting insights into what is happening in nature.

In his seminal paper, Kalleberg (1958) utilized large tanks and artificial stream channels to describe the territoriality of brown trout and Atlantic salmon through their juvenile ontogeny. In another influential work, Fausch (1984) utilized a large artificial stream section in the lab to investigate competition for the most beneficial foraging positions. Nevertheless, experimental arenas allowing for the natural home-range sizes of even young salmonids are difficult to harbour in the lab, and consequently, it will be difficult to completely replicate wild behaviour. A few studies have investigated dominance hierarchies directly in the wild. For instance, Jenkins (1969) and Bachman (1984) have produced descriptive studies of high value for the study of behaviour of stream living salmonids. In particular, these studies have provided important information about natural foraging patterns and how the social hierarchies influence microhabitat choice in open systems where the subject animals are free to move around. A more recent example is a study by Roy et al. (2013), which utilized PIT-tagging and a network of in situ short-distance antennae to show that there is a variety of activity strategies in Atlantic salmon parr, with some individuals being highly mobile in the river (called ‘wanderers’). The natural behaviour of wanderers is difficult to allow for in the lab, and behavioural estimates of competition behaviours obtained from lab-studies could likely be affected by this fact, to some extent.

Unrepresentative interactions in aggression studies

Aggression is associated with resource defence, typically through territoriality in stream-living salmonid fish (Kalleberg 1958; Keenleyside and Yamamoto 1962). Experiments on aggression typically use a live intruder fish [in full contact (Johnsson et al. 1999a; Tinoco et al. 2014), or enclosed in a separate chamber (Schjolden et al. 2005; Adriaenssens and Johnsson 2010)], a standardized model intruder (Dill et al. 1981), or a mirror image (Holtby et al. 1993; Johnsson et al. 2003; Höjesjö et al. 2004). Experimental ‘intruders’ should preferentially be physically enclosed to allow only visual and physical contact with the focal fish to reduce variation in aggression and avoid physical injuries. Still such intruders will inevitably vary in their expression of aggressive behaviour, which in turn will affect the response of the focal individual. While such effects are averaged

out when comparing groups of fish (e.g. Johnsson and Forser 2002; Filipsson et al. 2018), they are important to consider when evaluating among-individual variation in aggression. Variation in “intruder” aggression can be reduced by standardizing the size of the intruder relative to the focal fish. It is most common to use an intruder of somewhat smaller size than the focal fish to avoid inducing subordinate behaviour in the focal fish (Schjolden et al. 2005; Adriaenssens and Johnsson 2011), as relative size is used as a cue to fighting ability (Arnott and Elwood 2009; but see Huntingford et al. 1990).

Inanimate models pose a potentially good alternative, since they can be highly standardized; however, they lack the natural appearance and behavioural responses of a real intruder. These responses, which may include displays, colour changes, threatening poses, and chases and bites (Kalleberg 1958; Keenleyside and Yamamoto 1962) are used by the contestants to acquire information about the relative fighting ability of the opponent (Leimar and Enquist 1984; Enquist and Leimar 1990). Alternatively, relatively cheap options for displaying virtual fish animations, which could produce more realistic and standardized threat display, could be useful alternatives to physical intruders in laboratory settings (for more information, see e.g. Ingley et al. 2015; Chouinard-Thuly et al. 2017). However, for some species, like tilapia *Oreochromis mossambicus*, animations and videos appear to elicit different responses compared to real opponents (Wackermannova et al. 2017); how salmonids react to virtual stimuli remains to be tested.

The mirror image stimulation test (MIS) is another simple alternative to standardize the opponent, with the mirror image showing an equally sized contestant mimicking the behaviour of the focal individual (Gallup 1968). However, the test has been criticized for being unrepresentative of agonistic relationships in the wild (Ruzzante 1992). However, MIS aggression has been shown to correlate positively with dominance in several salmonid studies (coho salmon: Holtby et al. 1993; steelhead trout: Berejikian et al. 1996; brown trout: Höjesjö et al. 2004; but see Petersson and Järvi 2000). In our experience, however, MIS mainly elicit strong aggression in young juveniles, which may have to do with mirror images not being recognized as completely natural competitors in older fish (Bohlin et al. 2002a). In addition, there are potential problems with non-confrontative fish. The mirror-image will not

attack until the subject fish attack, and even if the subject can respond aggressively to being attacked, it will not show this agonism in a mirror test. Thus, there is a risk that MIS only measure willingness to escalate a conflict. Furthermore, even in the young and very aggressive fry, a mirror test requires some pre-thought before being conducted. Combining all aggressive act into a single score (e.g. time spent within a certain distance from the mirror), for instance, may not be a good idea, since a large proportion of the individuals tend to show aggression, which may lead to low variation in the collected data (Bohlin et al. 2002a; Näslund and Johnsson 2016b). Furthermore, aggression patterns in a group of fish often switches from direct attacks (active aggression) to displays (passive aggression) with time, as the individuals get familiar and establish a dominance hierarchy (Noletto-Filho et al. 2017). Instead, it is advisable to separate the different aggressive acts, at least into active and passive aggression (Johnsson et al. 2003; Näslund and Johnsson 2016b). Kalleberg (1958) and Keenleyside and Yamamoto (1962) are highly-recommended sources for categorizing the aggressive acts of salmonids.

Unrepresentative predator presentation in anti-predation studies

Prey responses are highly dependent on the behaviour of the predator (Lima and Dill 1990). Thus, whenever predation risk is investigated, it is advisable to standardize the predator stimuli in some way. Live predators can provide ecologically realistic scenarios and have been used successfully as stimuli in several studies on the anti-predator behaviour of salmonids (e.g. Johnsson and Abrahams 1991; Abrahams and Sutterlin 1999; Martel and Dill 1995; Höjesjö et al. 1999). However, using live predators will inevitably cause variation in the anti-predator response of the potential prey, reflecting variation in predator motivation. The variation in motivation among live predators (when several are used) can be reduced by acclimatising them well to experimental conditions, and by keeping their energy status (i.e. hunger level) as constant as possible, e.g. if a well-acclimated predator is kept hungry enough it is more likely to show a consistently high hunting activity (Ware 1966). It is also advisable to minimize size-variation among multiple predators in an experiment to avoid gape-

limitation effects (Godin 1997). Still, due to the problems discussed above and increasingly strict ethical regulations in many countries for using live predators in scientific studies, model predators are commonly used as an alternative (fish predator models: e.g. Einum and Fleming 1997; Fernö and Järvi 1998; bird predator models: e.g. Giles and Huntingford 1984; Höjesjö et al. 1999). However, models come with the opposite disadvantage of no added variation, that is, an ecologically unrealistic scenario since many prey species have evolved the ability to cope with variation in predator motivation and, consequently, risk (Lima 1998). A recent study on sticklebacks showed that the presentation of a model predator will, quite dramatically, affect the reaction by the subject “prey” individuals (Näslund et al. 2016). Thus, the selected presentation of a predator model will have potential influence on the interpretation of the results, and observations will only be valid for one specific, and often quite unnatural, predator encounter scenario. To make inference for wild animals, more information is needed. One approach is to use a range of different model presentation scenarios—different distances, different approach patterns, different model sizes, and different model species, to name a few possible variations. In addition, it might be a good idea to also actually utilize live predator individuals as a complementary stimulus treatment, to ascertain that the anti-predator behaviours observed when using the models do not deviate substantially from the responses to live predators. Another complementary experimental approach to using visible predators or predator-models is to utilize chemical cues from either predators, injured conspecifics, or both (see e.g. Chivers and Smith 1998; Brown 2003).

The problems of not using wild-type fish

Researchers commonly use hatchery-reared or domesticated, rather than wild fish in lab studies, because the former are easier to obtain in large numbers. Among salmonids, for example, domesticated rainbow trout are frequently used in behavioural studies, particularly in Europe where there are few naturally reproducing

populations, and the results are often extrapolated to be valid for wild salmonids as well (Gall and Crandell 1992). This assumption is problematic because domesticated rainbow strains have typically been selected for fast growth and associated traits (Tymchuk et al. 2009), sometimes for over a century (Gall and Crandell 1992), resulting in directional selection for behavioural traits positively correlated with rapid growth, like boldness, appetite, and aggression (Huntingford and Adams 2005). Behavioural traits are among the first traits to be affected by domestication selection, both advertently and inadvertently, so domesticated rainbow trout are likely to represent a deviating, and also narrower, range of behavioural phenotypes, as compared to the wild-type (Kohane and Parsons 1989; Sundström et al. 2004). For instance, overrepresentation of active, bold and aggressive behavioural types, i.e. a high-risk high-gain phenotype, are commonly observed (Johnsson 1993). This hypothesis is supported by the fact that interbreeding with domesticated rainbow trout increase risky exposure to predators in wild-type anadromous rainbow trout (steelhead) (Johnsson and Abrahams 1991). Moreover, domesticated rainbow trout suffer higher predation mortality in nature than conspecifics originating from wild parents (Biro et al. 2004). Consistently, the phenotypic traits of some strains of domesticated rainbow trout are more similar to genetically modified (for fast growth) rainbow trout than to the wild-type (Devlin et al. 2001; Tymchuk et al. 2009). Hatchery selection for fast growth may have altered the endocrinology of the domesticated rainbow to a level where the rainbow’s responses to hormonal alterations are not comparable with wild phenotypes. For instance, the food-intake response of domesticated rainbow to increased ghrelin levels is negative, while it is positive for most other species, including wild brown trout (Jönsson 2013; Tinoco et al. 2014). Thus, a large part of the range of behavioural phenotypes in wild populations, which also generally include more passive and shy strategies resulting from fluctuating and frequency-dependent selection in nature, are likely to be missing in many domesticated strains (Fig. 3; Huntingford and Adams

Fig. 3 Hypothetical distributions of behavioural phenotypes of a natural population (upper panel), and of a domesticated population (mid-panel). The lower panel shows an example of how selective capture methods may result in over- and under-representation of certain behavioural phenotypes in the sampled population. Note that whereas selective capture methods may lead to biased sampling of existing behavioural phenotypes, domestication may potentially eradicate some phenotypes from the natural population, as well as introduce novel phenotypes that were absent from the natural population. For simplicity, a normal phenotypic distribution is assumed here, whereas in reality behavioural distributions are often skewed which would alter the quantitative effects of unrepresentative sampling

2005). Importantly, domestication effects are detected already within the first hatchery generation, which means that any fish bred in artificial environments are behaviourally altered, as compared to wild conspecifics (Christie et al. 2012, 2016; Horreo et al. 2018).

Leaving the lab: studies in seminatural and natural systems

While lab studies have the major advantage of being highly controllable, they will inescapably lack in environmental realism (Fig. 4). Although some environmental features can be made more or less natural in the lab, it is impossible to provide salmonid fish (and most other animals) with a laboratory environment that matches natural conditions. Consequently, behaviour need to be studied in more natural environments to generate ecologically relevant answers. For this task, we identify four general different approaches that complements laboratory studies: (1) experiments in mesocosms, (2) experiments in closed natural systems, (3) experiments in open natural systems, and (4) monitoring in open natural systems (Fig. 4). Each of these approaches are exemplified with a couple of representative studies, for which pros and cons of the approach are discussed.

Experiments in mesocosms

Mesocosms are here defined as enclosed artificial experimental systems, which are replicable, and in which some key variables can be controlled and/or manipulated in a standardized way while still retaining many of the conditions present in a natural system. As such, a mesocosm can be a compromise between the highly controllable lab experiments and field studies (Fig. 4). Depending on the size of the enclosures, mesocosm studies may induce “fencing effects” causing unnatural behaviour due to restricted movements and dispersal, as well as possible elimination or reduction of predation risk (see e.g. Ostfeld 1994).

Example 1: effects of discharge on movement behaviour in Atlantic salmon

Our first example (Puffer et al. 2014), is a study utilizing outdoor mesocosms to investigate the influence of rapid fluctuations in water discharge (hydropeaking), caused by changes in electricity-demand from hydropower plants, on movement activity and foraging ability of Atlantic salmon juveniles. In this case, the mesocosms consisted of six parallel concrete stream channels (26 × 1.5 m), in which waterflow could be controlled (see Puffer et al. 2017 for illustration and photograph). Hatchery-reared salmon

Fig. 4 Different experimental systems, conceptually ordered along axes of precision of experimental data (as judged by how well external factors can be controlled during the experiment), and the realism (ecological relevance) of the results (as judged

by the restrictions the experimental system impose on the natural behaviour of the subjects). To illustrate that no experimental system is perfect on its own, a third scale of usefulness is drawn below

were tagged with passive integrated transponders (PIT), which could be detected by antennas through the channels to monitor the variation in movement behaviour in different flow-conditions. The results, in brief, indicated that salmon experiencing periodical hydropeaking increased their movements, which also negatively influenced their growth—a good example of environment-induced behaviour. The stream mesocosms represent a semi-natural environment, with natural food and natural cycles of light and temperature. The mesocosms also allow for short-distance movements, however, the fish are still confined within 26 m of a relatively shallow and narrow stream section. As such, this experiment provides a very useful insight into the variation of natural short-distance movements in the face of hydropeaking, but at the same time it lacks features of a larger stream (where powerplants are normally located), such as deeper pool areas and unconstrained possibility for downstream migration.

Example 2: habitat choice of juvenile Atlantic salmon under intercohort competition

The second example (Höjesjö et al. 2016), is a study from an indoor, glass-sided, semi-natural stream, divided into 16 consecutive mesocosms (0.9×0.6 m) by wire screens. Each mesocosm was landscaped into a shallow (≈ 12 cm) and a deeper (≈ 24 cm) area, and different numbers of age-0 and age-1 salmon were stocked into them to investigate habitat choice. Food availability was controlled. The results showed

that age-0 salmon adapt their habitat use and their foraging activity depending on whether older salmon are present. Like the first example above, this is a good indication of environment-dependent behaviour (this time relating to the social/competitive environment). Thus, the experiment provides insights into possible inter-cohort competition patterns. Nature-like stream flow and temperatures provide ecological relevance, but fish are again not able to freely leave the area of the mesocosm. Furthermore, natural food availability and predation was removed from the experimental setting. As in the first example, this is a good example of the necessary trade-off between experimental controllability and restrictions in natural behaviour. In contrast to the first example, the mesocosms are relatively small. This allows for higher level of replication at the cost of spatial restriction.

Experiments in enclosed natural environments

Taking a step from the controllable artificial mesocosms towards more realism, experiments can be conducted in enclosed natural environments. At smaller scale these enclosures could conceivably also be called 'mesocosms', as they may have largely overlapping features. However, in contrast to replicating mesocosms through a high degree of standardisation, replication of natural enclosures is carried out through randomisation of the enclosure location within the experimental area. Block-designs (i.e. pairing treatments close to each other to control for the variability) can be utilized, but replicates of

enclosures will typically vary to a higher degree than replicates of mesocosms, due to being parts of actual natural ecosystems. Natural enclosures may also be un-replicated when the experimentation is made on the individual level in larger enclosures (e.g. Johnsson et al. 1999b). The wide range of scales and level of controllability that can be applied in natural enclosures makes them overlap all other approaches, at least to some extent (Fig. 4). However, for each given setting the trade-off between precision and realism is omnipresent, as in all other approaches. Negative aspects of natural enclosures in running water include accumulation of debris on the fencing structures, which requires continuous cleaning (Fausch 2015) and accumulation of detritus inside the enclosures (Peckarsky and Penton 1990). In general, it is also difficult to obtain high replication levels in field experiments. Even if replication is not always necessary for scientific deduction (see Oksanen 2001), these limitations must be kept in mind when analysing the data (see Lemoine et al. 2016).

Example 1: behavioural flexibility in Arctic charr in response to social and environmental factors

This first example investigates how Arctic charr activity patterns are affected by fish density and environmental factors in an Icelandic stream (Fingerle et al. 2016). Four nylon mesh enclosures (4×1 m) were paired in two blocks (one high-density-, and one low-density treatment per pair) in a side-channel of a natural river. Natural food could pass through the mesh, but the natural substrate was covered by cobble stones to reduce environmental variation. Terrestrial predators were hindered by nylon strings stretched across the enclosures. The results showed that the charr adapt their activity depending on environmental factors such as light, temperature and discharge, as well as on the social environment, with activity being negatively influenced by light intensity, positively influenced by temperature and discharge, and generally elevated by a high population density. The enclosure being a part of the natural stream assured natural variation in the environmental factors. Just like in the examples of mesocosm experiments above, the movement restriction is unnatural, but it also allows for direct observation and behavioural scoring of individual fish over extended time-periods.

Example 2: habitat use and diet in brown trout

This second example describes a study where whole stream sections were fenced off in a forest stream (Giller and Greenberg 2015). Three enclosures (8×3 m), each containing a riffle section and a pool section were placed within a 200 m section of the stream, and ten individually PIT-tagged brown trout were released into each enclosure. Movements between the pool and the riffle were monitored by PIT-antennas located on the border between the habitats. Trout were classified into riffle-stayers, pool-stayers and movers, depending on their movement pattern in the enclosure. Based on this classification differences in diets could be determined, suggesting different foraging strategies being associated with habitat choice. This setup provides a nature-like stream setting that is extremely hard to mimic in artificial environments. The negative aspect is, again, that the trout were not able to move, should they have wanted to—but the confinement was the feature that allowed the researchers to follow individuals over time with high precision, with high likelihood of recapture. This study also highlights the potential variation among natural enclosures, since the enclosures themselves constituted significant factors predicting diet and growth of the trout.

Experiments in open natural systems

To achieve higher ecological relevance, experiments may be conducted in completely open natural systems. This step further reduces controllability of the experiment. Open systems mean that individuals are free to move around, are able to interact with natural competitors, predators, and prey. Both free movement and predation undoubtedly lead to missing individuals when it comes to collecting data. To some extent, loss of experimental subjects can be monitored by telemetry (e.g. by setting up antennas registering outmigration from the experimental area), but this solution is not completely fail-safe, as antennas sometimes malfunction. Experiments in open systems can be either manipulative, or natural. In manipulative experiments, the experimenter manipulates some feature of the individuals or their environment [e.g. by hormone implants (Johnsson and Björnsson 2001)] or the environment [e.g. fish density (Bohlin et al. 2002b), or stream flow (Fausch et al. 1997)], and randomly distributes individual subjects across treatments. In natural experiments, the researcher either

compare different populations with different characteristics or living in different environments, or survey populations after (and, if possible, before) a certain event has randomly occurred to a subset of otherwise roughly equivalent populations.

Example 1: foraging modes in different charr species

The first example is a short-term investigation into how changes in drift can affect foraging mode in charr species inhabiting mountain streams (Fausch et al. 1997). In this study, drifting food organisms were removed upstream from pools, in which naturally occurring Dolly Varden *Salvelinus malma* and white-spotted charr *Salvelinus leucomaenis* were observed while foraging. With the loss of drift, the subordinate Dolly Varden switched to benthic foraging, while the dominant white-spotted charr remained drift feeders. The usage of an open system (at least in the downstream direction, as a net catching drift was blocking upstream movements) in this case allowed for a natural expression of behaviour of the fish. Indeed, a substantial number of individuals were leaving the pool during the study. Forcing them to stay might have influenced the results since competition levels would have been higher. This type of observational studies is most suitable for short term studies, due to in- and outmigration of subject individuals, making continuity of observations difficult even if subjects are appropriately tagged.

Example 2: effects of rearing environment on large scale migration patterns in brown trout

A category of studies that require open systems are the investigations of how large-scale movement patterns are dependent on different factors. This example shows a study on how rearing environment affect migration performance in anadromous brown trout, by comparing wild and hatchery fish (Aarestrup et al. 2014). Both hatchery and wild fish were tagged with radio-tags and followed through their migration from the stream to the coastal areas of the sea. The study showed that rearing environment affect the migration behaviour as hatchery fish, naïve to the natural environment, were found to migrate faster. The hatchery fish were also showing higher mortality rates than the wild fish, suggesting poor anti-predation behaviour. It is obvious that a similar study, with natural conditions and predators, would be hard to realize in controlled environments,

especially for the marine part of the migration. However, the tracking of the migration and the losses of fish require high efforts, which are associated with some degree of uncertainty. Furthermore, variation in behaviours such as anti-predation behaviours must be inferred indirectly from survival rates.

Monitoring in open natural systems

Monitoring, in this case, refers to observations and measurements of unconstrained individuals in their natural environment, without any manipulation of the experimental system. This can be conducted in different ways. One way is a purely descriptive approach where the result is a data set from which we can construct hypotheses to be tested in future projects (Fig. 5). Another way is to construct a priori predictions and then investigate whether wild fish follow these predictions in nature (see *Example 1*, below). This method is inherently very vulnerable to confounding environmental factors, but at the same time, as close to natural conditions as possible, since little or no manipulation of the experimental system is imposed. Typical ways to investigate behaviour in open systems are snorkelling (Thurrow 1994; Nakano 1995), or stream-bank observations (Bachman 1984; McLaughlin et al. 1992) on stationary fish, or telemetry studies on either stationary or moving fish (Thorstad et al. 2013) (note that different methodologies cause different fright bias and differ in detectability of salmonids in streams, see e.g. Ellis et al. 2013). The monitoring approach can be very valuable when conducted over a gradient of different environments to follow-up and validate experimental results obtained in manipulative experiments (see e.g. Baxter et al. 2007; Fig. 5).

Example 1: seaward migration in brown trout fry

On Gotland, a large island in the in the brackish Baltic Sea, there were suspicions that some brown trout individuals actively migrate seawards already as fry, in contrast to the observation in the case of our previously told student-advisor story explaining smolt migration (Fig. 2). To test this hypothesis, which would provide evidence of higher behavioural flexibility in brown trout migration than previously thought, Järvi et al. (1996) decided to collect and investigate the fry found in the outlet of a stream on the

Fig. 5 Schematic illustration of the multi-faceted approach to research on salmonid behaviour. Each project is based on prior information (e.g. observations or previously published materials) which is used to formulate questions, which are translated to hypotheses, on which predictions are based. The hypotheses are tested in several experimental systems (black-bordered boxes; also see Fig. 4) and/or in several contexts (overlapping or non-overlapping in features) within each system. The results are obtained and evaluated based on the predictions to assess the

hypotheses. After evaluation, hypotheses may be reformulated for another round of experimentation; this is also the stage where sound results should be made public. The work-flow may also include non-experimental steps (grey-bordered boxes) to obtain novel information, such as systematic reviews to identify gaps in the current knowledge, meta-analyses to assess the robustness of the body of results available, and theoretical modelling to generate predictions from hypotheses

Gotland coast. The hypothesis that migrating fry could be actively moving downstream was supported by the fact that the collected fry at the coast had equal size and condition as the stream resident fry and, thus, were not necessarily the “losers” in early life competition (Elliott 1990). This study thus provided indirect indications of even larger variation in migratory behaviour of brown trout fry, than previously had been assumed. This behaviour is likely an adaptive response to the specific conditions on Gotland where streams sometimes dry out in summertime, and the

strategy can only be successful due to the low salinity of the Baltic Sea.

Example 2: dominance and ecology of wild red-spotted masu

In this observational study, Nakano (1995) classified individuals of the red-spotted masu *Oncorhynchus masou ishikawae* as either territorial or non-territorial, and whether they were dominant or subordinate. Three natural stream pools of the Hirakura Stream, Japan,

were utilized as study areas and observations were made on the individuals inhabiting the pools (recognized based on their individual parr marks, spot-patterns and scars) using underwater observations, with the observer lying motionless in the downstream area of the pool. Based on the individual scores of the fish, the results showed that dominant fish took and maintained station at the best foraging sites, had higher food intake and consequently faster growth rates. Furthermore, this study gave insight into the dynamics of a completely natural dominance hierarchy, which would be impossible to attain in a manipulated system.

The importance of combined and variable approaches

Combining lab and field studies is resource demanding and requires a range of skills in both lab and field techniques. The main advantage of combined approaches is that the precision of the lab experimentation can be combined with the variability of the natural environment. Combined approaches can be conducted by utilizing either a single set of individuals in both environments, or by splitting a sample population into two sets: one lab-group and one field group. Both methods have pros and cons. A single set of individuals can be utilized to first obtain good measurements of the behavioural trait under investigation, through repeated and standardized trials, followed by release and monitoring of performance (e.g. mortality and growth rates) in the wild (e.g. Höjesjö et al. 2002; Závorka et al. 2017). However, laboratory housing may have carry-over effects, which influence future behaviour in the natural environment, especially if the acclimation time reduce the lab-housed fishes' competitive ability (e.g. through reduced growth rate), as compared to the uncaptured competitors in the wild. Hence, the single-set approach can alternatively start with monitoring in the wild, with subsequent capture and trait-scoring under controlled conditions in the lab (e.g. Wilson and McLaughlin 2007; Závorka et al. 2016). This second option is not optimal either, since it will not be possible to monitor e.g. mortality in the wild (as it is not possible to score the dead individuals). Also, transport between the lab and the field site may also result in unnatural levels of stress (Specker and Schreck 1980), affecting the fish performance

regardless of whether the lab-scoring comes before or after the field test. Using separate sets of individuals instead prohibit intra-individual comparisons, and the comparisons must be made on the population level. Nevertheless, this methodology can provide good indications of whether lab experiments represent what happens under less controlled conditions. It is also the only option when regulations prohibit release of fish housed in captivity. When the latter restrictions apply, lab studies combined with closed mesocosm studies can be a useful alternative when inter-individual measurements are required (see e.g. Cote et al. 2017).

Example 1: circadian activity and dispersal of different behavioural types of brown trout

When aiming to investigate the performance of different behavioural types within a species, a combined approach with standardized behavioural scoring in the lab and performance monitoring in the natural environment suits the purpose well. In this example (Závorka et al. 2016), brown trout were tagged, immediately released close to their capture site (to avoid breaking the dominance hierarchy in the system by removing individuals), and then monitored for circadian activity using portable RFID antennas. Thereafter, the fish were recaptured, brought to the lab and scored for activity in an open-field test. After release back into the stream, the fish were monitored for circadian activity again. This approach allowed for investigating both the effect of behavioural type (on a scale from more to less active) on the circadian activity of the trout, and how the different types respond to being removed from the stream for a number of days. Results showed that fish scored as less active in the lab were mainly detected in the stream at night, while more active fish were detectable over the whole diel cycle. It also showed that more active fish dispersed more after re-release into the stream after lab-scoring.

Example 2: effects of anxiety drugs on migration behaviour of Atlantic salmon

Here a combined-approach was used to investigate the migration-willingness in Atlantic salmon after being exposed to an anxiety-reducing drug, commonly found in wastewater (Hellström et al. 2016). The study was first conducted on one set of individuals in circular runways in the lab, where movement along with the

current was assumed to signal downstream sea-ward migration. A second set of exposed salmon smolts were then stocked into a small stream, where their outmigration was monitored by PIT-telemetry. Both studies showed that drug-treated fish migrate to higher extent than unexposed fish. Thus, the results suggested that the drug could potentially be used to improve seaward migration in hatchery-produced smolts.

The importance of considering the scale of the study

The final example in the previous section appeared robust, however, while the results from the natural stream indicated successful migration of the drug-treated fish, a follow up study in a larger river showed that the treated fish were not successful migrants at all (Hellström et al. unpublished). In the larger river, the altered behaviour led to predation by northern pike *Esox lucius*, a species not present in the smaller stream. This complementary larger-scale experiment nicely illustrates the importance of also varying the context and scales of experiments within the lab and the field, to cover as many different scenarios as possible. In the lab it may be possible to vary the tank-size, social environment, resource availability, and environmental complexity in standardized ways to achieve more robust conclusions, while in the field it may be possible to conduct experiments in different spatial and temporal scales, as well as in streams differing in certain specific characteristics. Another general take home message from these studies is that premature conclusions from field experiments also can have implications for decision-making, for example in fisheries management. Further presentation of issues relating to both spatial and temporal scales are discussed in Folt et al. (1998).

Synthesis: validating the ecological and evolutionary relevance of behavioural studies through multi-level questions and the multi-faceted approach

Highly standardised protocols make it easy to compare results across studies, but also comes with the risk of continuous misrepresentation of some environmental variable which may consistently influence the realism of the results in the ecological context. As noted by Shrier (2005): “[...] replication of all aspects of the

study is more likely to yield consistent results, but this does not necessarily mean true results. Since we don’t know a priori which methodological details are most appropriate [...], heterogeneous results from different designs is an important source of information and can lead to a new, more in-depth understanding of the subject [...]”. For some types of research, high standardization is highly recommendable, while it is not necessarily so for others. Behavioural ecology is arguably a field where results are highly context dependent, and hence a variable approach among experiments can be very useful to produce appropriate conclusions. This is particularly important when dealing with highly plastic species like salmonids, which can vary enormously both among and within populations (e.g. Jonsson and Jonsson 2011; Klemetsen 2013). Variable experimental procedures will have a higher chance of encountering interesting phenomena and exceptions from general patterns, which might be relevant for a better understanding of the behavioural ecology of the investigated species. In general, variable procedures also have more potential to address behavioural questions at multiple (ultimate and proximate) levels, as discussed earlier. This is not a call to abandon standardization across experiments, but an acknowledgement of the practical usefulness of variable approaches to achieve progress in knowledge. By mixing lab-, mesocosm-, and field studies, the chance of finding inconsistencies in the results will increase. In comparison to only doing one type of experiments, and only finding highly consistent results, the multifaceted approach may lead to further scientific progress (Fig. 5). Finding that the lab and field tests of the same hypothesis do not yield the same conclusions is an advancement of knowledge, and something to continue to build upon in the aim to find out how nature works.

Even if a research-team is limited in their opportunities to vary the experimental setting between lab and field conditions, a multifaceted approach is still possible, through conceptual replication within the available facility. Conceptual replication means that the same question is investigated (in the same species) by differently designed experiments, i.e. by changing the context in which the experiment is run (Kelly 2006). This approach is also suitable to show the level of consistency in experimental results within lab, mesocosm and field conditions. While exact replication is the gold standard for reliability of experimental results, and especially important when previous

studies has had low sample sizes or noisy data (Loken and Gelman 2017), it leads only to narrow-sense replicability (Heard 2017)—it only shows that the experimental results are valid given a specific experimental context. A suite of conceptual replications can provide broad-sense replicability, and stronger evidence for the conclusions, if they are all in line with each other (Heard 2017). When enough empirical studies have been carried out (and, of course, reported!), further validation can be obtained by meta-analyses, or, if studies are too diverse, by narrative reviews and syntheses (Fig. 5).

Planning experiments based on a multi-faceted approach (i.e. varying experimental setups in the lab as well as in the field) will either facilitate the achievement of broad-sense replicability and firm conclusions, or lead to the identification of context-

dependent mismatch of the results. Both results are highly important for the advancement of knowledge. By investigating the multiple levels of causation, provided by Tinbergen's four questions (Fig. 2) and its associated theories, through a multi-faceted approach of investigation (Fig. 5), we gain a very robust basis for multi-level thinking about behavioural variation in salmonids (and other animals).

Open questions for future research

Determination of the most important questions for future research efforts is a somewhat subjective endeavour, and each researcher should be free to approach this from her/his own perspective and interest. Nevertheless, in Box 1 we present ten broad

Box 1 Ten suites of important questions to further understand causes and effects of behavioural variation in salmonids

In this box we present ten suites of questions to be answered to reach a fuller understanding of variation in salmonid behaviours. Each suite is accompanied by a few references that, at least partly, touch upon these questions or provide further information about how to approach them. Many questions are complementary to each other and could be combined within research programmes.

- 1 *Genetic versus non-genetic determination of behavioural phenotypes:* How strong modifiers are environmental and parental effects in comparison to the direct genetic effects determining the behavioural type and flexibility of an individual? (e.g. Aubin-Horth and Renn 2009; Ledón-Rettig et al. 2013; Burton et al. 2013; Bengtson et al. 2018)
- 2 *Integrated phenotypic traits:* How is (are) the full covariance pattern(s) among physiological, behavioural, and life-history traits expressed? Do such trait syndromes vary depending on ecological context? What is the hierarchical structure among different behavioural traits? And does the syndrome-structure vary within and among populations? (e.g. Berman et al. 2016; Závorka et al. 2017; Dammhahn et al. 2018; Montiglio et al. 2018)
- 3 *Plasticity over ontogeny:* Are individuals consistently different through life, i.e. through the fry, parr, smolt, post-smolt, and adult stages? If not, what determines changes in behavioural characteristics and what is the scope for change? (e.g. Groothuis and Trillmich 2011; Edenbrow and Croft 2011)
- 4 *Selection pressures on risk-taking:* Does selection for and against bold-like behaviours differ through ontogeny due to the large differences in the habitat of different life-stages? If so, what are the strongest selective forces on the population level? (e.g. Werner and Anholt 1993; Biro et al. 2004; Näslund et al. 2017a)
- 5 *Vulnerability to anthropogenic factors:* Do human exploitation and alterations of the environment affect different behavioral phenotypes in different ways? Are there certain human-caused ecological traps inadvertently targeting certain phenotypes? (e.g. McLaughlin et al. 2013; Mittelbach et al. 2014; Arlinghaus et al. 2017)
- 6 *Population vulnerability:* Does behavioural variation in a population buffer against exploitation and disastrous events, e.g. through differences in boldness or migration patterns? And does the proportion of different behavioural phenotypes in a population affect the extinction risk of the population? (e.g. Mittelbach et al. 2014; Pruitt and Modlmeier 2015)
- 7 *Cascading ecosystem effects:* Does the proportion of different behavioural phenotypes in a population affect the ecosystem, e.g. through different food preferences? If so how strong are the cascading effects? (e.g. Sih et al. 2012; Boukal 2014; Des Roches et al. 2018)
- 8 *Stocking and aquaculture escapes:* Do stocked fish and aquaculture escapees alter the behavioural variation in natural populations, e.g. through interbreeding or competition? And if so, what are the fitness consequences for the affected natural populations? (e.g. Einum and Fleming 2001; Chittenden et al. 2010; Skaala et al. 2012; Cote et al. 2015)
- 9 *Macroevolutionary patterns:* Do different salmonid species differ in their behavioural traits, behavioural variation, and trait-associations? And how strong is the phylogenetic signal in determining these characteristics? (e.g. Garamzegi et al. 2012; Sol et al. 2018; Mayhew 2018)
- 10 *Experimental methodology:* What do different experimental trials actually measure? How consistent are different trial setups supposedly measuring the same trait? And does laboratory measurements reflect behavioural expression in nature? (e.g. Burns 2008; Carter et al. 2013; Laskowski et al. 2016)

suites of questions concerning behavioural variation in salmonids that, in our opinion, deserves to be investigated further. Working with these suites of questions will not only increase the knowledge about salmonid behaviour, but will also advance the fields of ethology and behavioural ecology in general.

Concluding remarks

By now we hope it is evident that it is a long way to go from observing a trout fry responding to a novel object (Fig. 1), or a salmon smolt swimming down a river in spring (Fig. 2), to thoroughly understanding all aspects of the observed behaviour, including why it is so variable between individuals. Hopefully, however, the keen salmonid behaviour ecologist has not been discouraged by all the challenges discussed in the previous sections, but rather found inspiration and ideas about how to address all the fascinating behavioural puzzles that salmonids and other fish have to offer. If so, this is the time to put down this paper, go to the lab, and to the field, and study the fish to extend our knowledge for the benefit of current and future researchers, managers, and stake-holders.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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