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The role of ecological context and predation risk-stimuli in revealing the true picture about the genetic basis of boldness evolution in fish

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Abstract To showcase the importance of genotype × environment interactions and the presence of predation risk in the experimental assessment of boldness in fish, we investigated boldness in terms of feeding behavior and refuge use in two genetically different populations of juvenile carp (*Cyprinus carpio*) in two replicated experimental conditions in ponds and laboratory tanks. The populations were expected to exhibit genetic differences in boldness due to differential evolutionary adaptation to low-predation-risk pond aquaculture conditions. Boldness was measured in variants of open-field trials with and without implementation of additional predation risk-stimuli by angling on feeding spots. Without explicit implementation of risk, genotypes adapted to low-risk environments, i.e., domesticated mirror carp behaved consistently bolder than their less domesticated scaled conspecifics in the pond environment, but not in the laboratory environment. When we implemented artificial risk-stimuli by angling on previously safe feeding spots, boldness differences among genotypes also emerged in the laboratory environment, indicating strong genotype × environment effects on boldness behavior of carp. The expected genetic basis of boldness differences among genotypes was clearly supported in the pond environment, while the laboratory study revealed these patterns only under inclusion of explicit risk-stimuli. Our study thus underscores that boldness may involve both a basal component that is expressed independently of obvious predation risk (e.g., in open fields) and a component revealed in relation to explicit predation risk, and both dimensions may respond differently in behavioral tests.

Keywords Genotype × environment interactions · *Cyprinus carpio* · Predation risk · Common garden · Angling

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Introduction

Evolutionary adaptation of life-history traits in response to predation-induced selection pressures is well documented in several taxa ranging from insects, over birds, and fish (Sæther 1988; Reznick et al. 1990; Gotthard et al. 1994). It has also been commonly reported that behavioral traits vary consistently within and between animal populations in response to the level of predation risk (Seghers 1974; Cousyn et al. 2001; Stoks et al. 2003; Ghalambor et al. 2004; Herczeg et al. 2009). Such adaptation includes behavioral traits commonly summed under the temperament trait "boldness" (Herczeg et al. 2009). In fish, boldness—defined as the individual's reaction to any risky, but not new situation (Réale et al. 2007)—is expressed in behaviors like use of risky habitats (Wilson and McLaughlin 2007), exploration and activity (Wilson and Godin 2009), foraging under risk of predation (Wilson and Stevens 2005), or schooling (Seghers 1974). In line with the hypothesis that boldness-related traits should evolve in response to predation-induced selection pressures (Seghers 1974), populations of fish adapted to low-predation-risk conditions were found to exhibit greater

risk-taking behavior than fish adapted to high-risk conditions (e.g., Seghers 1974; Magurran et al. 1992; O'Steen et al. 2002; Ghalambor et al. 2004). The main explanation put forward for these findings is that too bold behavior can be disadvantageous in the presence of predators by increasing the probability of deadly attacks, such that the average boldness of a population of fish should be lower in high-predation environments relative to low-predation conditions (Seghers 1974; Brydges et al. 2008).

Studies on genetic adaptation of behavioral traits can be challenging and have mainly been pursued by a comparative approach where populations supposed to be adapted to different predatory regimes have been compared, often using fish as a model species (Brydges et al. 2008; Conrad et al. 2011). When properly conducted, differences in average boldness among populations adapted to different levels of predation risk can support inferences about an underlying genetic basis of behavioral phenotypes. Such inferences are particularly strong if examinations of adaptation of behavioral patterns to predation risk are conducted using common-garden reared animals under laboratory conditions (Kawecki and Ebert 2004). Indeed, many studies on boldness differences among populations of fish have used common-garden reared offspring and subsequently applied laboratory-based boldness assessments (e.g., O'Steen et al. 2002; Herczeg et al. 2009). Some comparative studies on boldness differences among fish populations used individuals directly collected in the wild (Magnhagen 2006; Archard and Braithwaite 2011). These studies reported that fish from high-predation-risk environments exhibited greater (rather than lower) risk-taking behavior compared to individuals collected from low-predation-risk environments (e.g., Brown et al. 2005; Magnhagen 2006; Archard and Braithwaite 2011). However, in the absence of common-garden designs, study findings may well be explained by plasticity rather than genetically based evolutionary adaptation (Conover 1998; Kawecki and Ebert 2004).

Common-garden studies can provide stronger inferences about the potential genetic basis of behaviors, but such studies are not free of biases when behavioral assays are conducted in laboratory contexts (Nuismer and Gandon 2008). This is due to uncontrolled effects of the artificial assessment environment on the test animals and their phenotypic expressions (Kawecki and Ebert 2004). A range of laboratory effects may potentially explain conflicting findings in earlier among-fish population comparisons in terms of boldness-related behaviors. For example, Bell (2005) failed to identify expected differences in swimming activity outside refuges between common-garden reared offspring of three-spined stickleback (*Gasterosteus aculeatus*) populations with different predation backgrounds, when trials were conducted in a novel laboratory environment that lacked explicit predation-stimuli. By contrast and being consistent with expectations, the fish supposed to be genetically adapted to high predation risk were indeed found to be more timid than those adapted to low-risk conditions when observed in the presence of a predator. Although not specifically discussed by Bell (2005), this study highlights the potential for genotype × environment interactions in experiments when behavioral responses of genetically different animals across various environments or situations are observed. Moreover, in behavioral tests, different components of genetic adaptation of the complex trait "boldness" might be measured, and any subdimensions of the supposed overarching boldness construct may have evolved different responses in relation to predators. For example, in the population studied by Bell (2005), local behavioral adaptation to predation risk might not have happened on the basal level of behavior (e.g., swimming activity in the absence of obvious predation risk), but rather on the behavioral response to predation risk, which would subsequently only be expressed under test conditions including risk-stimuli. Consequently, behavioral phenotypes revealed in experimental trials by fish may be strongly affected by genotype × environment interactions and the presence or absence of predation-stimuli, highlighting the importance of standardized experimental setups when researchers aim to identify phenotypic differences between differently adapted populations. Otherwise, study findings, particularly regarding the genetic basis of observed behavioral differences and generality of these findings, need to be treated with caution.

In a second example on the difficult issue of inferring the genetic origin and the exact portion of the boldness axis revealed through laboratory experiments on comparative boldness differences among fish populations, Brown et al. (2007) found laboratory-reared *Brachyraphis episcope* derived from parents from high-predation sites to emerge significantly faster from a shelter than lab-reared fish derived from low-predation parents. These results were derived using classical open-field tests designed to neutrally measure boldness in fish. Following common expectations (Seghers 1974), too bold behavior should have been outselected under high predation risk such that one would have expected fish from high-predation sites to emerge more slowly from shelter compared to fish from low-predation sites. Yet, no predation risk-stimuli were implemented during the open-field tests by Brown et al. (2007). It is thus unclear whether the basal boldness expressed by the study animals in the open field would have been different in the presence of more explicit predation risk-stimuli as highlighted by the study by Bell (2005). Indeed, the neutral open-field test within the laboratory, as applied by Brown et al. (2007), was only designed to reveal basal differences in boldness and did not aim at testing alternative traits under selection such as predator recognition or response to explicit predation risk. Because all of these traits together are characteristic for the complex composite trait "boldness," the true underlying differences in boldness among populations should ideally be tested to cover a greater range of plausible behavioral reactions of fish exposed to varying predation regimes in the wild. Our

examples highlight the necessity for explicitly accounting for the impact of predation risk-stimuli to isolate the effects of the genotype, the environment, and genotype × environment interactions on boldness-related behavioral comparisons among fish populations that are supposed to be evolutionarily (i.e., genetically) adapted to predation risk.

To elucidate the impact of the assessment environment (pond or laboratory environment) along with the inclusion of predation risk-stimuli on among-population differences in boldness, in the present study, we compared the expression of boldness-related traits among two juvenile carp (*Cyprinus carpio* L.) genotypes reared in common-garden prior to experimentation. The two populations were differentially adapted to low-predation-risk pond aquaculture conditions and should therefore differ in average boldness. This is because farmed fish have been consistently found to be bolder relative to less domesticated fish (Berejikian 1995; Huntingford 2004; Huntingford and Adams 2005; Conrad and Sih 2009). Therefore, we would expect our carp populations to consistently differ in average boldness in an open-field test in the laboratory and in the more natural pond environment, both in the basal boldness as well as in their response to explicit risk of predation.

Material and methods

Our experiment was designed to measure how two genotypes of common-garden reared carp with known differences in adaptation to predation risk differ in their expression of three boldness-related traits (number of visits at two different feeding spots and intensity of sheltering) in a non-novel environment and to assess whether there is an impact of the assessment environment and of artificially induced predation risk-stimuli on boldness expressions of the fish within the different environments. First, behavior of the fish was tested in three replicated ponds. The pond environment did not contain any natural fish predators, but offered latent predation risk through fish-eating birds and potentially through olfactory cues from predators like pike (*Esox lucius*), because the ponds were continuously supplied with water from a large natural lake with known existence of several fish predators (Lewin et al. 2004). Moreover, the ponds represented an environment comparable to the original evolutionary environment of the test fish (e.g., farm ponds used for aquaculture). In a second step, the same boldness-related traits were measured in a situation where artificially induced predation risk-stimuli were implemented through standardized angling on feeding spots to test for the effects of artificial risk-stimuli on boldness expressions of the study fish. To further investigate the role of risk-stimuli for the expression of adapted differences in boldness and further to remove any potentially confounding factors that might have existed in the outdoor ponds, similar replicated experiments were conducted within a large laboratory tank without any kind of predation risk-stimuli except for the standardized angling tests. Again, fish were first observed without any risk-stimuli, followed by observations during implementation of risk. In this way, we were able to experimentally test for the effects of the assessment environment (semi-natural in ponds vs. laboratory) and the effects of artificially induced predation risk-stimuli (i.e., angling on feeding spots) within the two different environments on boldness expressions of fish with known differences in adaptation to risk.

Study animals

Among fish, pond-cultured carp exhibit the longest history of artificial selection starting about 2,000 years ago (Balon 2004). Distinct carp genotypes and phenotypes have developed as a consequence of adaptation to suites of low-predation-risk pond conditions (Steffens 1980; Balon 2004). The most obvious phenotype indicating differential degree of artificial selection and adaptation to pond environments is the scale pattern of common carp, which can be broadly distinguished into scaled and mirror phenotypes (Balon 1995). Scaled carp are fully scaled, reflecting the original morphotype of wild common carp, whereas mirror carp have much less scales, reflecting the morphotype that is strongly domesticated and highly adapted to low-risk pond conditions (Probst 1953; Balon 2004; Matsuzaki et al. 2009). All carp used in our study were raised at a commercial fish hatchery (Fischzucht Wegert, Ostercappeln, Germany; 52°19′52″ N, 8°14′48″ E) in the same common-garden pond environment. Parental fish descended from two selection lines: (1) a selection line with scaled morphotypes and (2) a selection line in which scaled morphotypes were previously crossed with strongly domesticated mirror carp selection lines. Fish from both selection lines were stocked into the same common-garden pond for reproduction. Young-of-the-year mirror carp could only develop as a result of two breeders from the strain originally crossed with domesticated mirror carp (strain 2) (Kirpichnikov and Billard 1999). All juvenile carp were exclusively fed with standard carp dry food in addition to any natural food ingested in the shallow (1.5 m deep) earthen common-garden pond (40 m× 50 m). At an age of 10 months, the fish were transported to the Leibniz-Institute of Freshwater Ecology and Inland Fisheries in Berlin, Germany. There, fish were kept in tanks (1 m×1 m× 1 m; 5 fish per 100 l) with tap water (mean temperature±SD 18±1.5°C, exchange rate once per day). Fish were fed with standard carp pellets (5 mm diameter, Aller Classic, Aller Aqua, Golßen, Germany), and the total daily food amount was ~1.5% of the fish body wet mass. Before the behavioral experiments started, fish were slowly acclimatized to water temperatures within the test environments (see the following discussion) by altering the temperature at a maximum of 1°C per day.

Tagging of fish

All carp (N=100 scaled carp and N=100 mirror carp) were individually marked with passive integrated transponders (PIT) to observe fish behavior using PIT antenna systems. We surgically implanted PIT tags (23 mm length, 2 mm width, Oregon RFID, OR, USA, 2% tagging mortality) into the fish's body cavity following the method described by Skov et al. (2005). Before PIT implantation, fish were anesthetized using 1 ml l^{-1} of 9:1 solution of ethanol:clove oil in well aerated water at 18°C. After PIT implantation, all fish were measured for total length (TL, to the nearest 1 mm) and wet weight (to the nearest 1 g).

Behavioral experiments under pond conditions

Stationary passive telemetry systems within three replicated experimental ponds (12 m×5 m×1 m; $L \times W \times H$; Fig. 1) were simultaneously used to enumerate carp behavior by two genotypes in ponds in September 2008. To investigate boldness parameters, each of the three ponds was stocked with 40 similar-sized carp (20 scaled carp and 20 mirror carp, mean TL ± SD pond 1: 199±6.9 and 199±12.1 mm, T-Test, t=0.08, P=0.936; pond 2: 199±4.7 and 200±11.9 mm, T-Test, t=−0.26, P=0.797; pond 3: 199±6.1 and 197±11.2 mm, T-Test, t=0.78, P=0.440), which were allowed to acclimatize for 2 days before behavioral observations started.

The ponds were continuously supplied with water from the nearby Müggelsee in Berlin (800 ha; shallow; eutrophic; 52°26′57″ N, 13°38′59″ E). Inflow into the ponds was about 1 ls^{-1} unfiltered lake water. Ponds were carefully cleaned

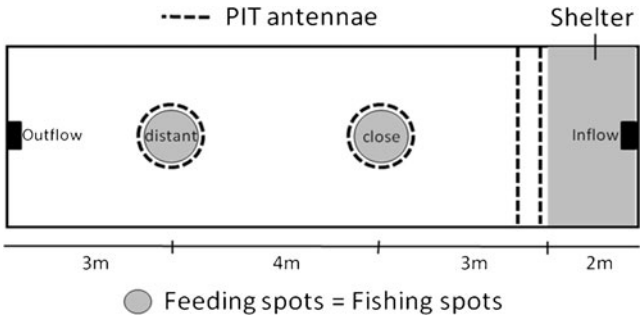

Fig. 1 Setup of the passive telemetry system used in the laboratory and ponds. We installed four antenna loops recognizing individual fish passages through the shelter entrance or when visiting the feeding spots. Two of the antenna loops were installed in front of the shelter to reveal the swimming direction of the fish from subsequent recordings. Another two antennae were used to cover the feeding spots. These antennae consisted of a circle and were placed at the bottom, providing detections of fish directly entering the circle. All data collected by the system were stored on a datalogger (Internal datalogger board, Texas Instruments, Dallas, TX, USA) and downloaded daily. Food was provided at the middle of the round feeding spots. When artificial predation risk was implemented, the baited hook was also placed in the middle of the feeding spot circle

before the experiment, and the bottom was covered with clean gravel (diameter 2–5 mm). Despite the absence of fish predators in the ponds, sources of predation risk in the pond environment were present through fish-eating birds regularly visiting the outdoor ponds and potentially through the presence of olfactory cues by predatory fish introduced into the ponds through the inflow of Müggelsee. Fish in the ponds were thus assumed to be permanently faced with some degree of latent predation risk, reflecting the generally mild latent risk-conditions present in commercial carp aquaculture ponds, i.e., the environment that the study animals have adapted to in the past.

The ponds contained a shelter structure (2 m×5 m) made out of black plastic material just above the water surface next to the water inlet and close and distant feeding spots (0.5 m diameter each) in different distances to the shelter (Fig. 1). All of these three structures were covered by PIT antennae (Fig. 1) so that we were able to quantify the individual number of visits at the feedings spots and the time spent sheltering as three measures of boldness. Because fish were allowed to acclimatize within the pond setup, the environment was not new to the fish, yet potentially risky, and behavioral measurements were thus considered indicative of boldness (Réale et al. 2007), but not indicative of exploratory behavior (Réale et al. 2007). The shelter structure was assumed to be perceived by the fish as the safest habitat, but lacking abundant feeding opportunities. Thus, foraging was only possible by taking the risk of leaving the shelter structure. All fish entering the feeding spots had to cross the large open pond area, comparable to variants of an open-field test (Budaev 1997). Open areas are more attractive to bold fish (Sneddon 2003), and we assumed bolder fish to show increased presence at the feeding spots. Fish were fed on the feeding spots on seven consecutive days to determine the foraging activity in the absence of any experimentally induced predation risk. This provided an initial measure of boldness. Feeding started 2 h before sunset until 2 h after sunset to control for potential impacts of daytime on boldness measurements. Feeding was conducted on a 60-min basis, alternating between the two feeding spots. Standard carp pellets (5 mm diameter, see previous discussion) were used as food, and the total daily food amount of pellets was 1% of the fish body wet mass at the time of stocking. In addition, for every single pellet, one sweet corn (5–7 mm diameter, Bonduelle, Reutlingen, Germany) was offered to also provide novel, yet preferred food for carp (Klefoth et al., unpublished data).

To test for potential behavioral changes of scaled and mirror carp in response to artificial predation risk, angling was conducted for another seven consecutive days. Angling was assumed to be perceived by the fish as a standardized, neutral, and mild form of artificially induced predation risk because learned hook avoidance as a consequence of hooking and subsequent live-release has been documented in

carp (Beukema 1969; Raat 1985). Angling took place simultaneously to the daily feeding sessions and on the same spots. Sweet corn was used as bait, provided on a bold-rig as described by Rapp et al. (2008). This method ensured exclusive shallow hooking of the fish. The hook was connected to a 13-cm multifilament soft leader. The angling equipment consisted of a 3-kg monofilament line and a short fishing rod. Bites were indicated by an electronic bite indicator (Carp-Sounder Basic VR, Carp-sounder, Germany). After hooking, the fish was landed quickly using a small rubber net to prevent mucus abrasion (Barthel et al. 2003). Fish were then placed into a bucket filled with fresh water for unhooking and PIT identification (Pocket reader, Allflex, Dallas, TX, USA). Afterwards, fish were immediately released in the middle between the two feeding spots. Release of the fish was always conducted within 30 s, and no mortality occurred. The whole experimental procedure within the pond environment lasted 14 consecutive days (7 days of feeding without angling-induced risk, followed by 7 days of feeding under angling-induced risk).

The environmental conditions in the ponds were documented using temperature loggers (TidbiT datalogger, Onset, Bourne, MA, USA) and using data from a weather station located 500 m away from the Müggelsee, providing data on an hourly basis for wind speed (m s^{-1}), global radiation (W m^{-2}), light intensity (μmol m^{2-1}) at 0.75-m water depth, air pressure (mbar), humidity (%), and air temperature (°C). Mean water temperature ± SD in the ponds over the study period was 19.0±0.5°C (range 17.0–20.2°C).

Behavioral experiments under laboratory conditions

We conducted an additional laboratory-based experiment under controlled environmental conditions to test for the consistency of boldness differences among the two carp genotypes under fully controlled conditions. This experiment resembled the setup established in the ponds and used a new set of study animals (see previous discussion). Experiments were conducted in a large laboratory tank (10 m×4 m×1 m; $L \times W \times H$) of comparable size to the ponds. The tank was connected to a circulating water system and a biological filter. Water inflow was 2 $l s^{-1}$, and water temperature ± SD was constant at 22±1°C. To investigate boldness parameters in the laboratory, a total of 40 similar-sized fish (20 scaled carp and 20 mirror carp, mean TL ± SD 225±20.0 and 229±16.0 mm, respectively, *T*-Test, $t=-0.80$, $P=0.441$) were stocked into the tank and allowed to acclimatize for 2 days before behavioral observations started. Behavioral experiments followed the same protocol described for the pond experiment and assessed the same behavioral variables of boldness, with the exception that the intervals of changing the feeding spots within the daily 4-h feeding periods were 15 min instead of 60 min for logistical reasons. After the 7th day of angling within the laboratory environment, experimental carp were replaced by a new set of 40 fish (20 scaled carp and 20 mirror carp, mean TL ± SD 224±17.0 and 229±21.0 mm, respectively, *T*-Test, $t=-0.89$, $P=0.377$), and the experiment was replicated.

Data recording and statistics

From the raw PIT data, we calculated the boldness parameters "time spent sheltering" (min h^{-1}) and "number of visits at the feeding spot" (# h^{-1}), with the latter separately for the close and distant feeding spots. We defined a fish to be sheltering after it had passed the PIT antennae in front of the shelter (Fig. 1) in a direction from the outside to the inside of the shelter. Sheltering activities ended when the fish passed the antennae in the opposite direction. If a fish was not detected at both antennae, sheltering ended when the fish was observed to be elsewhere than the shelter. Visits at the feeding spots were defined by single observations of individual fish. To prevent overestimation of visits by multiple detections within a short time frame in which the fish did not leave the feeding spot, an interval of 30 s was applied before a new visit was counted. Pretest experiments showed that the maximum time fish spent within the circle antennae was always less than 30 s (Klefoth et al., unpublished data), justifying our assumption. Because the number of visits at the close and distant feeding spots was highly correlated (Spearman's rho =0.88, $p<0.001$, in the pond environment, and Spearman's rho =0.78, $p<0.001$, within the laboratory environment), the mean number of visits at the close and distant feeding spots per individual and per unit time was used for subsequent analyses, resulting in a single variable describing the number of visits at the feeding spots. No such strong correlations were found between the number of visits at the feeding spots and the time spent sheltering (all Spearman's rho <0.5). Therefore, the time spent sheltering was used as an additional boldness-related parameter.

Functionality of the PIT system was tested in a first trial prior to conducting the pond and laboratory studies with different fish within the same general setup. This was done because stationary PIT systems are known to be limited in their ability to detect multiple individuals at the same time (Zydlewski et al. 2001). We assessed the behavioral measures—"time spent under shelter" and "number of visits at the feeding spots" estimated from the raw PIT data—and tested for correlations with visual observations of the same parameters. Results using Spearman correlations between observed and calculated data for the time spent under shelter for 10-min periods (Spearman's rho =0.474, $P=0.030$, $N=18$) and the number of visits at the feeding spots during 10-min periods (Spearman's rho =0.575, $P<0.001$, $N=36$) confirmed

a high functional capability of the PIT system to remotely measure boldness-related traits of carp (Klefoth et al., unpublished data). All calculations of boldness parameters were conducted for feeding and non-feeding periods for every fish on a daily basis (two data points per fish and day). To standardize for differences in the duration of feeding periods (4 h) and non-feeding periods (rest of the day), mean values per hour were calculated.

We used Generalized Linear Mixed Models (GLMM) to explain sheltering activities (min h^{-1}) and mean visits at the feeding spots (# h^{-1}) in the pond environment and the laboratory. Fish ID nested within pond or tank replicate was added as random factor to account for repeated measures and the nested structure of the experiments. We used the dataset to test for differences in the behavioral response of scaled and mirror carp (Genotype) to food supply without risk (Feeding) and the period when artificial predation risk was implemented while feeding (Risk). Individual TL was added as covariate to all models because no differences between within and between subject effects were identified when centering TL within ponds as outlined by van de Pol and Wright (2009). Previous capture and release events (Capture) were considered in the model as well to control for potential impacts on subsequent behavior of the fish (Klefoth et al. 2008; 2011). All possible two-way and three-way interactions with Genotype, Feeding, and Risk were added to the models. In all cases, data were overdispersed, and a quasi-Poisson error distribution was found to be the best fit to the data. We used the software package R and the Penalized Quasi-Likelihood method (function glmmPQL) in library MASS (R Development Core Team 2009). Variances explained by the models were calculated using the "predict method for glmmPQL," also provided in library MASS. Predicted values were regressed against observed values using linear regression.

To account for uncontrolled environmental conditions in the ponds, we conducted a PCA with varimax rotation on all environmental data collected, generating two components [variable and factor loading, respectively: global radiation (0.971), light intensity (0.870), humidity (−0.885), air temperature (0.984) (PC1, explained variance: 57.1%, eigenvalue: 3.4); wind speed (−0.545), air pressure (0.802) (PC2, explained variance: 17.3%, eigenvalue: 1.1)], together explaining 74.4% of the total variance. In initial models, the estimated variance components of factor scores for PC1, PC2, and water temperature were generally low (< 6%). Therefore, environmental parameters were removed from further analyses.

Results

Comparisons of boldness-related behaviors between scaled and mirror carp strongly differed between the two ecological contexts studied (Figs. 2 and 3). In the pond environment, the main effect of Genotype was found to consistently and significantly explain visits at feeding spots independent of ecological context. Importantly, no significant interactions between Genotype and the environmental factors Feeding or angling-induced Risk on feeding spots were present (Table 1; Fig. 2), underscoring the robustness of the genotypic differences in foraging behavior in the pond environment, even in the absence of natural fish predators. In line with expectations, the more domesticated mirror carp were found twice as often on the feeding spots compared to their less domesticated scaled conspecifics (Table 1; Fig. 2). In addition to Genotype, the fixed effects Feeding and Risk were also found to significantly affect foraging behavior in the pond environment. Accordingly, in periods where food was supplied on feeding spots, the number of visits by both genotypes was generally higher, and also under these conditions, mirror carp visited the feeding spots more frequently than scaled carp. For example, the mean number of visits ± SD at feeding spots while feeding and before implementation of angling-induced risk of mirror carp and scaled carp was 3.2±3.0 and 1.6±1.8 visits h^{-1}, respectively (Table 1; Fig. 2). Interestingly, during periods when angling-induced risk was present in addition to food on the feeding spots, fish in the ponds were more often found on the feeding spots compared to periods without risk (mean number of visits ± SD at feeding spots while feeding and after

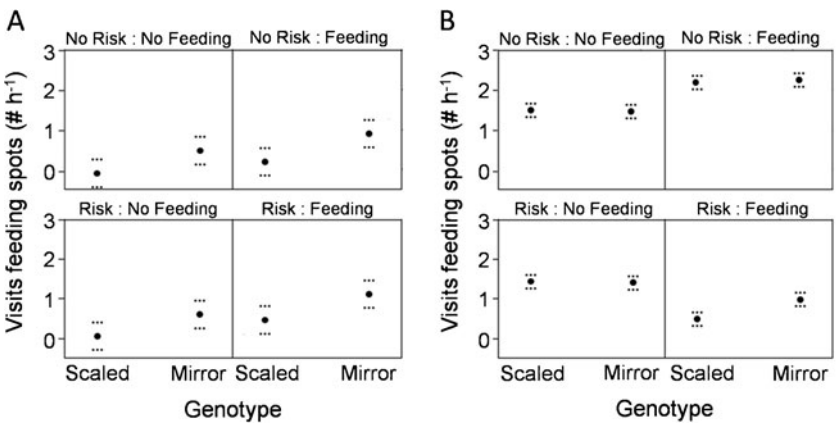

Fig. 2 Behavior of scaled and mirror carp in response to feeding and non-feeding times with and without implementation of risk through angling **A** in ponds and **B** in the laboratory. The figure shows the least squares means and the 95% confidence intervals of the mean number of visits at feeding spots (# h^{-1}). Bold p-values indicate significant effects

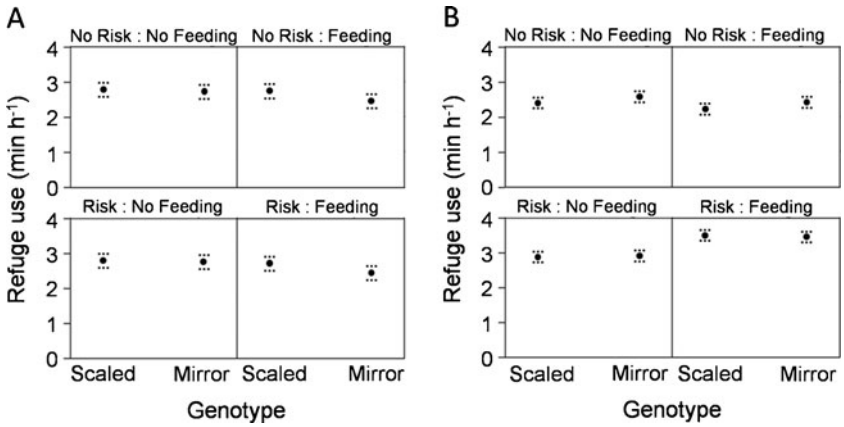

Fig. 3 Behavior of scaled and mirror carp in response to feeding and non-feeding times with and without implementation of risk through angling **A** in ponds and **B** in the laboratory. The figure shows the least squares means and the 95% confidence intervals of the time spent sheltering (min h^{-1}). Bold p-values indicate significant effects

implementation of angling-induced risk for mirror carp and scaled carp 4.0±3.5 and 2.1±2.4 visits h^{-1}, respectively, Table 1; Fig. 2). In addition to the aforementioned main effects, in the ponds, we also found the size of the carp to be positively related to the number of visits at the feeding spots (Table 1).

A different picture was evident in the laboratory, where the main effect Genotype was significantly interacting with the other main effects Feeding and Risk to explain the number of visits at feeding spots by scaled and mirror carp in a more complex manner (Table 1; Fig. 2). Therefore, the revealing of boldness differences of the two genotypes in terms of foraging was driven by the environment, and thus, it is context dependent. The provision of food in the absence of risk on feeding spots generally increased the number of food patch visits (Fig. 2). However, in contrast to the pattern in the pond, the onset of angling on previously risk-free feeding spots reduced the frequency of feeding spot visits by both genotypes in absolute terms when food was supplied in the laboratory. In fact, the mean number of visits declined by approximately 83% to 1.5±2.7 visits h^{-1} for scaled carp and by 73% to 2.6±3.5 visits h^{-1} for mirror carp after implementation of angling-induced risk, in turn reaching levels that were present in the pond already at the onset of the

Table 1 Nested Generalized Linear Mixed Models (GLMM) to predict the effects of Genotype, Feeding, Risk (induced by angling), and previous Capture and all relevant two-way and three-way interactions on the mean number of visits at the feeding spots (# h^{-1}) in the pond environment (*left*) and the laboratory (*right*). All models included individual fish nested within pond or laboratory replicate as a random effect. Total length was considered as covariate

Fixed effects	Estimate ± SE	DF	*T*	*P*	R^2	Fixed effects	Estimate ± SD	DF	*T*	*P*	R^2
Pond feeding spots					0.43	Lab feeding spots					0.48
Intercept	−4.21±1.84	2,605	−2.3			Intercept	1.23±0.36	2,011	3.5		
Genotype[a]	0.59±0.17	112	3.5	**<0.001**		Genotype[a]	−0.06±0.07	74	−0.8	0.403	
Feeding[b]	0.31±0.06	2,605	4.9	**0.001**		Feeding[b]	0.69±0.04	2,011	19.7	**<0.001**	
Risk[c]	0.16±0.07	2,605	2.5	**0.013**		Risk[c]	−0.06±0.04	2,011	−1.4	0.158	
Capture[d]	−0.20±0.24	2,605	−0.8	0.405		Capture[d]	−0.23±0.09	2,011	−2.5	**0.012**	
Length	0.02±0.01	112	2.2	**0.028**		Length	0.00±0.00	74	0.8	0.438	
Genotype × Capture	−0.13±0.27	2,605	−0.5	0.629		Genotype × Capture	0.08±0.11	2,011	0.7	0.502	
Genotype × Feeding	0.13±0.08	2,605	1.6	0.112		Genotype × Feeding	0.12±0.05	2,011	2.3	**0.023**	
Genotype × Risk	−0.03±0.08	2,605	−0.4	0.723		Genotype × Risk	0.03±0.06	2,011	0.4	0.683	
Feeding × Risk	0.09±0.08	2,605	1.0	0.302		Feeding × Risk	−1.63±0.07	2,011	−23.9	**<0.001**	
Genotype × Feeding × Risk	−0.01±0.11	2,605	−0.1	0.916		Genotype × Feeding × Risk	0.40±0.09	2,011	4.4	**<0.001**	

[a] Reference is scaled carp

[b] Reference is non-feeding times

[c] Reference is period without angling-induced risk

[d] Reference is not being captured previously

experiment in the absence of predation risk and subsequently during food supply (Fig. 2). In contrast to the situation in the pond, in the laboratory, the number of visits at the feeding spots was almost equal for both genotypes before angling started, both within and outside feeding times (Fig. 2), and it remained so after angling started in periods lacking food supply. However, the use of feeding spots diverged between the genotypes once fishing started in periods when food was supplied, and the visits were then found to be, on average, 67% higher for mirror carp relative to scaled carp. These combined results indicate that there were indeed genetically based differences in the propensity to forage among scaled and mirror carp, but the revealing of these differences in the laboratory was strongly context dependent and only occurred under conditions of food provision and the presence of predation risk. In addition to Genotype, Feeding, and Risk, previous capture events also affected the future number of visits at the feeding spots by reducing their frequency in the laboratory (Table 1).

In terms of sheltering activities, a few significant effects were found to predict refuge use within the pond environment, and in contrast to the foraging behavior, no significant context-independent main effect of Genotype was present. However, in line with expectations, mirror carp spent significantly less time under shelter compared to their scaled conspecifics when food was supplied, as revealed by a significant Genotype × Feeding interaction (Table 2; Fig. 3), and this pattern was unaffected by the presence or absence of angling-induced risk (non-significant Genotype × Feeding × Risk interaction, Table 2; Fig. 3). This result indicates that mirror carp behaved generally bolder in terms of refuge use during food supply within the ponds than scaled carp, irrespective of the existence of more explicit angling-induced risk-stimuli.

The findings on sheltering were less conclusive in the laboratory. Both genotypes similarly increased sheltering activities when food was supplied during the periods of angling-induced risk compared to feeding in the absence of angling (significant Feeding × Risk interaction, Table 2; Fig. 3). In terms of differences among genotypes, refuge use by scaled carp increased in a somewhat more pronounced fashion in response to the onset of angling-induced risk relative to the similarly expressed average increase in sheltering shown by their mirror carp conspecifics, resulting in a significant Genotype × Risk interaction. However, absolute and relative differences among genotypes in terms of refuge use in the laboratory were small and statistically independent of feeding (non-significant Genotype × Feeding × Risk interaction, Table 2; Fig. 3), although descriptively they were most clearly expressed in the absence of feeding. These results overall reveal that the generally small differences in sheltering activities between scaled and mirror carp

Table 2 Nested Generalized Linear Mixed Models to predict the effects of Genotype, Feeding, Risk (induced by angling), and previous Capture and all relevant two-way and three-way interactions on the time spent sheltering (min h^{-1}) in the pond environment (*left*) and the laboratory (*right*). All models included individual fish nested within pond or laboratory replicate as a random effect. Total length was considered as covariate

Fixed effects	Estimate ± SD	DF	*T*	*P*	R^2	Fixed effects	Estimate ± SD	DF	T	P	R^2
Pond shelter structure					0.19	Lab shelter structure					0.38
Intercept	2.64±0.88	2,605	3.0			Intercept	2.46±0.27	2,011	9.1		
Genotype[a]	−0.07±0.09	112	−0.8	0.445		Genotype[a]	0.18±0.06	74	2.8	**0.006**	
Feeding[b]	−0.05±0.05	2,605	1.1	0.281		Feeding[b]	−0.18±0.05	2,011	−3.3	**<0.001**	
Risk[c]	−0.01±0.05	2,605	−0.2	0.847		Risk[c]	0.48±0.05	2,011	10.2	**<0.001**	
Capture[d]	0.17±0.2	2,605	0.8	0.402		Capture[d]	−0.04±0.07	2,011	−0.6	0.568	
Length	0.00±0.0	112	0.2	0.868		Length	−0.01±0.00	74	−0.2	0.841	
Genotype × Capture	0.01±0.24	2,605	0.0	0.979		Genotype × Capture	0.06±0.08	2,011	0.7	0.472	
Genotype × Feeding	−0.21±0.07	2,605	−2.9	**0.004**		Genotype × Feeding	0.02±0.07	2,011	0.3	0.768	
Genotype × Risk	0.04±0.07	2,605	0.6	0.581		Genotype × Risk	−0.14±0.07	2,011	−2.1	**0.035**	
Feeding × Risk	−0.01±0.07	2,605	−0.2	0.836		Feeding × Risk	0.79±0.07	2,011	12.1	**<0.001**	
Genotype × Feeding × Risk	−0.04±0.10	2,605	−0.4	0.684		Genotype × Feeding × Risk	−0.10±0.09	2,011	−1.1	0.289	

[a] Reference is scaled carp

[b] Reference is non-feeding times

[c] Reference is period without angling-induced risk

[d] Reference is not being captured previously

within the laboratory mainly depended on the implementation of angling-induced predation risk and that there were no differences in behavioral expressions of scaled and mirror carp regarding their basal level of refuge seeking in the laboratory in the absence of risk.

Discussion

Our study revealed the expected difference in boldness-related behaviors among two genotypes of differently domesticated carp in terms of a higher average boldness of the more strongly domesticated mirror carp relative to scaled carp, particularly in relation to feeding behavior in the presence of predation risk by angling on feeding spots and to some degree also in relation to refuge use as a second dimension of boldness. However, this conclusion was only unambiguous when the two genotypes of carp were tested under pond conditions. In the laboratory, the addition of artificial predation risk to the test trials was needed to more clearly reveal boldness differences among the two carp populations, and again, this was mainly the case for feeding-related behaviors and less pronounced for refuge use. The importance of tests conducted in tank versus pond environments and the impact of predation risk-stimuli on study outcomes in the laboratory indicated the existence of genotype × environment interactions as it relates to boldness expressed by genetically distinct populations of carp. Simply applying a variant of a classical open-field test, which is often assumed to reliably measure boldness in fish (e.g., Budaev et al. 1999; Brown et al. 2007), in a large laboratory tank would thus have provided inconclusive or even misleading results in terms of adapted boldness differences among the two carp strains. Most importantly, one would probably not have concluded a genetic basis of boldness differences among the two carp genotypes using a laboratory experiment alone, without addition of artificial predation risk to the behavioral assay. This is because an open-field test without additional risk-stimuli under laboratory conditions may not separate the effects of the genotype from potentially important genotype × environment interactions. Furthermore, by only applying open-field tests without behavioral observations under risk, one may not be able to distinguish between adapted behavioral responses towards predation risk and genetic adaptations of basal boldness as, for example, measured by exploration of a non-novel, yet potentially risky open area between the refuge and the feeding spots, and our study underscores that carp genotypes may express these traits differently depending on ecological contexts. The general similarity of our tank and pond experimental setups (e.g., both were lacking fish predators) gave rise to important differences in study findings as it relates to boldness differences of carp. We raise to mind to not prematurely discard the possibility for genetic adaptation of fish populations in terms of boldness, even if this pattern is not immediately revealed in an open-field laboratory study that controls all other potentially "confounding" environmental factors. Potentially, one then needs to implement some form of predation risk to reveal genetic variance in non-basal dimensions of boldness.

Our study showed large effects of the ecological context (pond vs. laboratory environment) and ecological factors (existence of predation risk-stimuli within the laboratory environment) on the expression of boldness-related traits of two genotypes of carp that were expected to generally differ in basal boldness due to genetic adaptation to low-risk aquaculture conditions. Because consistent and context-independent behavioral differences between scaled and mirror carp were only found in the pond environment, our study underscores earlier recommendations on the design of comparative studies in fish if these are aimed at revealing the genetic adaptation of behaviors to key local ecological factors. Either such studies are to be conducted using common-garden reared offspring in the laboratory, which allows the removal of confounding environmental variation and the "clean" testing of individual environmental factors (e.g., risk of predation or food supply). However, as our study showed, experimenters may not reveal the true picture of boldness adaptation if the correct environmental stimuli are missing. An alternative perspective may be, in light of the lack of clear boldness differences among our carp strains, that selection has not been strong enough to change basal boldness expected to be expressed in an open-field test in the absence of explicit predation risk (Brown et al. 2007). However, we contend that studies on genetic adaptation of behavior should also be conducted under less controlled conditions by exposing test populations to a range of natural environmental factors supposed to be involved in their evolution. If technically feasible, such studies may be conducted within the original evolutionary environment using reciprocal transplant approaches (Kawecki and Ebert 2004; Walling et al. 2004) or in ecological conditions reasonably close to those of the original evolutionary environment. We contend that the experimental ponds that we used in our carp studies represented a reasonable approximation of the original evolutionary environment, and maybe not surprisingly, the differences in boldness among mirror and scaled carp were robust and clear in this pond environment, even in the absence of any additional predation risk (e.g., also in basal levels of boldness).

Results of our study suggest that when comparative population studies on the genetic variance of boldness of fish are conducted in the laboratory, careful choice of the ecological context and the appropriate predation risk-stimuli may be needed to reveal robust results. Ideally, the laboratory may also mimic the original environmental conditions as close as possible (Kawecki and Ebert 2004), although researchers should keep in mind that with increasing complexity of the experimental setup, uncontrolled environmental effects or complex genotype × environment interactions might complicate study results. Thus, standardized experimental protocols

and setups allowing isolation of the behavioral responses of interest should generally be favored over trying to mimic nature in laboratory environments. Thereby, the benefits of laboratory trials (designed to isolate cause and effects) may ideally be combined with the strength of more natural environments (designed to study individual responses to a suite of correlated or uncorrelated natural factors) using common-garden reared individuals if studies are indented to reveal patterns of local adaptation. We urge, however, to be careful about implicating about the lack of genetic adaptation if laboratory results do not reveal the expected patterns. One might have missed to include the appropriate test stimulus or generally measured the wrong trait that has not been under divergent selection in nature.

Our findings showed that consistency of boldness-related traits in fish can be impacted by the presence or absence of stressful situations like those induced by predation risk. Earlier studies have shown that randomness of behavioral expressions tends to be predominantly pronounced in non-threatening situations (Alados et al. 1996; Budaev et al. 1999), and in our study, the lack of boldness differences among carp strains in the absence of angling in the laboratory shows that the large open field was likely not perceived as threatening by the fish, presumably facilitated by rapid learning and habituation as no other predators were present in the fully controlled laboratory tank. Therefore, we contend that one should attempt to measure several dimensions of boldness in laboratory studies to avoid inappropriate conclusions based on a restricted set of measures that may capture different dimensions of the composite trait boldness. Thus, the internal validity of boldness-related measures under laboratory conditions should be highest by incorporating several different measures of boldness, including observations under predation risk (Toms et al. 2010), thereby considering potential interaction effects of the genotype and the environment (Gerlai and Csányi 1990) and also distinguishing between adaptation of boldness-related traits on the basal level (as, for example, revealed in open-field tests) and in relation to more explicit risk of predation (as, for example, revealed in our experiment by using angling on previously safe feeding spots).

In the pond environment, we revealed mirror carp to be generally and consistently bolder than scaled carp. Though no natural predatory events were observed during the study period, the presence of fish-eating birds in the pond area was observed repeatedly—a factor that is known to influence the foraging behavior and sheltering activity of fish (Allouche and Gaudin 2001). Sources of latent predation risk in the pond environment might have also been based on olfactory cues by predatory fish despite the absence of fish predators in the experimental ponds. This is because all ponds were provided with water from a large natural lake, potentially containing chemical cues from predatory fish such as pike. Aquatic animals evolve sensitive receptors for detecting these cues for the assessment of predation risk (Wisenden 2000), and prey can smell chemical cues of their predators, even if they have never encountered the predator (Chivers and Smith 1998; Kats and Dill 1998). We thus assume that the existence of latent predation risk was responsible for consistent differences in boldness among our carp populations, also in the absence of artificial predation risk by angling. This conclusion was reinforced by our laboratory findings, where boldness differences among carp populations were only evident when risk-stimuli were introduced into the experiment and where the number of visits at the feeding spots and the time spent sheltering significantly changed after implementation of risk. Furthermore, in the laboratory, the number of visits of carp at the feeding spots reached comparable levels to those within the pond environment, but only when predation risk in the form of angling was introduced. This suggests that the less frequent use of feeding spots in the pond compared to the laboratory in the absence of angling may reflect the "standard" behavior of carp when latent predation risk is present.

Generally, our findings, particularly those from the ponds, were in agreement with a wide range of other studies comparing the behavior of common-garden reared fish from high-and low-predation sites in the laboratory (e.g., Huntingford and Wright 1992; Magurran et al. 1992; Bell and Stamps 2004; Ghalambor et al. 2004). In line with our results, all of these studies showed that fish adapted to low-risk conditions were, on average, bolder than their high-risk conspecifics when faced with artificially implemented or natural risk-stimuli. Opposing findings in the literature (Brown et al. 2007) might be related to locally different selection pressures or have a methodological cause by only measuring boldness-related traits on the basal level and omission of tests with more explicit risk of predation. Moreover, fish tend to exhibit high plasticity in terms of expression of behavioral phenotypes (Dingemanse et al. 2010; Stamps and Groothuis 2010) such that testing of wild-captured fish with a life-time experience in a high-predation environment may exhibit greater boldness compared to low-predation conspecifics. The most robust information about the genetic basis of behavioral traits can be expected by using common-garden reared fish (Kawecki and Ebert 2004), and thus, we consider our findings on the differences in boldness among our two carp strains to have a genetic origin.

In addition to the importance of common-garden protocols, our study also highlights the importance of considering potential effects of genotype × environment interactions in laboratory protocols designed for among-population comparisons of boldness in fish. However, in much of the current fish behavioral literature, the importance of standardized risk-stimuli in assessments of boldness seems to be underappreciated, and various researchers employ different predation-stimuli in their boldness tests (Toms et al. 2010; Conrad et al. 2011), potentially influencing study outcomes. The absence of a standardized experimental protocol for boldness-related measurements also constrains the comparability of studies and may affect the

reliability of study findings in potentially important ways. Brown et al. (2007) argued that perception of predation threats might differ between fish adapted to high-risk and low-risk conditions. This makes it difficult to distinguish if observed behavioral differences in the presence of predators are based on adapted differences in boldness or adapted differences in threat recognition, in turn motivating the use of open-field tests as a clean measure of basal boldness of fish (Brown et al. 2007). However, as our laboratory experiment has shown, genetic variance in basal boldness in an open-field test may only emerge in the presence of latent predation risk. Thus, the expression of basal boldness might be a function of the perception of some level of predation risk, and open-field tests might not necessarily offer this degree of functionally important level of risk, at least not within the laboratory in common carp. Thus, to generate robust findings in studies on adaptation of populations, we recommend inclusion of different setups, including different behavioral measurements with and without explicit risk to more fully elucidate the genetic adaptation of the behavioral repertoire of fish populations to predation risk in the wild. In this way, the effects of genotype, environments, and genotype × environment interactions can be better understood, leading to an improved understanding of the adaptive divergence of the focal trait.

We choose to implement angling on feeding spots as an experimental inclusion of predatory threat to avoid using real predators or predator models, thereby circumventing the issue of differential threat recognition evolution to natural predators (Brown et al. 2007). We assumed angling to constitute a neutral risk-stimulus as hook avoidance learning was previously documented in carp angled in pond environments (Beukema 1969; Raat 1985). The fact that we could observe behavioral alterations towards angling-induced risk only within the laboratory environment (as indicated by a reduced frequency of visiting feeding spots) suggested two implications. First, angling was perceived as threatening in the laboratory, leading to a reduced usage of feeding spots, and the level of threat for carp in the pond environment was not strong enough to further reduce a basal level of visits at feeding spots. The very similar level of visits on feeding spots per individual and hour was also reached in the laboratory after angling started, collectively indicating that while angling was surely perceived as a threat, feeding spots did not completely lose their attraction to our study fish.

We found that the experience of previous capture significantly reduced the number of visits at the feeding spots in the laboratory. This can be explained by learning effects as described previously (Beukema 1969), which may have been more pronounced in the cognitively simpler tank environment (Girvan and Braithwaite 1998) and be facilitated by greater water clarity that may have helped carp to identify angling gear and avoid being hooked. However, capture was also a covariate in the pond model so that any capture-related effects on boldness-related behaviors were statistically controlled, and the overall study findings were robust.

There were few differences in the experimental setup between the tank and pond environments, and this might have influenced the study findings (e.g., different temperature, environmental exposure, and water clarity). Moreover, fish used in the laboratory approach were slightly larger in size than those used within the ponds. However, size of the fish did not differ between the two genotypes in any of the ponds or tank replicates. In addition, we used total length of individual fish as a covariate in our statistical models, and thus, we are certain that any behavioral differences between scaled and mirror carp on the population level were not caused by the size of study animals. However, we found a significant and positive relationship between the size of the fish and the number of visits at the feeding spots within the pond environment. It has been repeatedly shown that the basal levels of boldness in fish are independent of the size of the fish (Sundström et al. 2004; Brown et al. 2005), but instead larger size attained by a given fish can be a consequence of bold behavior (Johnsson 1993). Because our fish were raised within a natural pond with regular food supply prior to experimentation, among-individual differences in size might correlate with boldness and related higher feed intake rates, potentially explaining why larger fish were more often found on the feeding spots within the pond environment.

In conclusion, our study revealed interactions between the genotype of carp and the ecological environment in which boldness was measured. The genetic basis of boldness differences among the two populations of carp was unambiguous in the more natural pond environment, even in the absence of fish predators and angling-induced predation risk. Similar behavioral differences between our two genotypes of carp were also found in the laboratory when tested under risk of predation, highlighting the potential for adapted behavioral responses towards predation risk rather than basal boldness expressions per se. Due to the common-garden approach, our study provides evidence about genetic adaptation of boldness in carp (particularly in response to predation risk). From a methodological perspective, our study underscores the suggestions by Kawecki and Ebert (2004) that robust local adaptation studies should ideally be conducted under natural conditions or in laboratory conditions involving a range of experimental stimuli. Reciprocal transplant studies in the wild are one possible way for the future that can also take advantage of modern tracking technologies like PIT systems, as applied in our experimental study. This may also help in eliminating the potential for observer bias effects through remote observation of individual behavioral patterns. In this way, evolution of behavioral traits in response to different predator regimes or other ecological factors can more realistically be studied without the potential for experimentally induced complications through genotype × environment interactions that may lead to erogenous conclusions. Alternatively, boldness-related measures

under laboratory conditions should incorporate various boldness measures, including observations with and without explicit predation risk, thereby considering potential interaction effects of the genotype and the environment and also distinguishing between adaptation of boldness-related traits on the basal level and in response to explicit predation risk.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical note Animal handling associated with this study was approved through an animal care permit (*No G 0178/09*) granted by the State Office of Health and Social Affairs in Berlin in accordance with the German Animal Protection Act.

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