The bright, the bold and the toxic: do coloration, personality, and toxicity represent an integrated phenotype in fire salamanders?

Max Mühlenhaupt[1]*, Henry J. Bohny[1], Maria Moiron[2,3], Manuela Schmidt[1], Laura Schulte[1], Barbara A. Caspers[1,3]

[1] Department of Behavioural Ecology, Bielefeld University, Konsequenz 45, 33615 Bielefeld, Germany

[2] Department of Evolutionary Biology, Bielefeld University, Konsequenz 45, 33615 Bielefeld, Germany

[3] Joint Institute for Individualisation in a Changing Environment (JICE), Bielefeld University and University of Münster, Konsequenz 45, 33615 Bielefeld, Germany

* Corresponding author, E-mail address: max.muehlenhaupt@uni-bielefeld.de

Abstract

Defensive coloration such as bright colors used to advertise secondary defenses (i.e., aposematic coloration) is very common but also shows high intraspecific variation. Similarly, consistent among-individual differences in behavior (i.e., animal personality) are pervasive in the animal kingdom. Therefore, aposematism and personality could be linked to produce an optimal defensive phenotype, however, this has not formally been investigated. Here, we used the European fire salamander (*Salamandra salamandra*) to study if personality traits correlate with an individual's proportion of yellow and relative toxin gland size using open field tests and observations during husbandry. Four of the five tested behaviors showed low to moderate

but significant repeatabilities. However, only the activity during husbandry showed a positive correlation with the relative toxin gland size indicating a potential trade-off between foraging and the costs of chemical defenses. Furthermore, three of the four personality traits showed strong correlations between them, and all personality traits were higher in fire salamanders collected in fall compared to spring, indicating the importance of seasonality effects on fire salamander personality. While we found little evidence for a potential role of trait integration maintaining individual variation in behavior and coloration of fire salamanders, future studies on personality traits in aposematic species should consider the potential of covariation of personality with coloration and/or toxicity.

Significance Statement

Variation in warning coloration is prevalent in many aposematic species but represents a paradox nonetheless given the strong selection on this trait. Similarly, animal personality has been identified in different aposematic species but a dedicated test of the covariance of personality traits with warning coloration and/or toxicity has not been conducted. This study has tested the potential of trait integration of personality, warning coloration and toxicity in fire salamanders. We believe this research will motivate future studies on other aposematic species and can open a fascinating new field intersecting the research on warning coloration and animal personality.

Keywords: Aposematism, Repeatability, Amphibians, Chemical Defenses, Behavioral Syndrome, *Salamandra salamandra*

Introduction

Organisms use a variety of traits to decrease the risk of being predated, which can involve morphology, behavior, and physiology (DeWitt and Langerhans 2003). These functionally

related traits can show strong correlations among and within individuals to compensate or complement each other in order to produce an effective phenotype that is referred to as an "integrated phenotype" (Pigliucci 2003; Klingenberg 2008; Murren 2012). A common example are chemically defended animals that display strongly contrasting colors to advertise secondary defenses to potential predators, an anti-predator mechanism known as "aposematism" (Rojas et al. 2015; Caro and Ruxton 2019; Ruxton et al. 2019). Despite strong selection on the warning coloration, many aposematic species show high intraspecific variation in the characteristics of the coloration that advertise their chemical defenses (Briolat et al. 2019). This represents a selective paradox given that avoidance learning in predators should be maximized if the signal is uniform (Mappes et al. 2005; Briolat et al. 2019). However, early life experiences can have an effect on the warning coloration of aposematic species later in life (Sanchez et al. 2019), the coloration can incur maintenance costs (Grill 1999; Ohsaki 2005; Friman et al. 2009; Caspers et al. 2020; Barzaghi et al. 2022), or can be involved in other functions such as mate choice (Rojas et al. 2018; Briolat et al. 2019) or thermoregulation (Briolat et al. 2019). Therefore, different mechanisms can pose potential sources of intraspecific variation in warning coloration. Furthermore, the integration with other traits relevant in an anti-predator context such as specific behaviors can complement a given aposematic phenotype. For example, Rojas et al. (2014) showed that aposematically colored frogs show different movement behavior in accordance with different color patterns and thereby complement a color trait with a behavioral trait to optimize their escape strategy.

Behavioral ecologists have been studying the causes and consequences of repeatable individual differences in behavioral traits, known as "animal personality" (Dingemanse and Réale 2005; Réale et al. 2007; Stamps and Groothuis 2010) for over two decades. It is apparent that animal personality is pervasive in the animal kingdom (Bell et al. 2009), and has important consequences for an individual's fitness (Dingemanse and Réale 2005; Smith and

72 Blumstein 2008; Wolf and Weissing 2012). For example, bolder, more active individuals can
73 achieve higher growth rates and more reproductive events through more frequent foraging and
74 mating opportunities (Werner and Anholt 1993; Smith and Blumstein 2008), but are also at
75 higher risk of mortality due to more frequent predator encounters (Lima and Dill 1990 but see
76 Moiron et al. 2020; Haave-Audet et al. 2022). Given the trade-off between maximizing
77 reproductive output and mitigating mortality risk, one focus of animal personality research is
78 on the correlation with other traits (e.g., morphology or physiology) in order to understand
79 how individual variation evolved and is maintained (Sih et al. 2015). A meta-analysis by
80 Niemelä and Dingemanse (2018) revealed a positive, but weak association between
81 personality traits such as boldness, exploration, and activity with non-behavioral traits such as
82 metabolic rate, hormone levels, and body size across the literature. The authors suggested that
83 one reason for this weak association could be interactive effects of multiple traits (i.e.,
84 integration of many different traits). Therefore, multiple functionally related traits should be
85 considered when studying the causes of individual differences.

86 Given the paradoxically high variation in warning coloration of many aposematic species, it is
87 surprising that to the best of our knowledge the relationship between personality, color and
88 toxicity has not been thoroughly studied in any aposematic species. Variation in coloration
89 and/or toxicity could influence personality traits through negative feedback (e.g., because
90 warning coloration and toxicity are costly to produce and maintain, an individual with
91 relatively high expression of these traits needs to be bolder to increase food intake (Werner
92 and Anholt 1993)) or positive feedback (e.g., because an individual is better protected from
93 predators due to its high expression of warning coloration and toxicity, it can “afford” to be
94 bolder (Sih et al. 2015)). While plenty of evidence of repeatable individual differences of the
95 behavior of aposematic species exists (e.g., Kelleher et al. (2017); Cossio et al. (2024); Klank
96 et al. (2024)), the relationship with coloration or toxicity have not been investigated yet. In the

aforementioned study by Rojas et al. (2014) a functional relationship in aposematic frogs between color pattern variation and movement behavior was reported but repeatability of the behavior was not confirmed. Similarly, associations between toxin gland size, coloration and locomotor performance have been shown in natterjack toads (*Epidalea calamita*) but behavioral repeatability has not been confirmed (Zamora-Camacho and Comas 2019). In the non-aposematic but nonetheless chemically defended American giant millipede (*Narceus americanus*), latency and duration of conglobation (considered as boldness traits) showed repeatability but were not associated with an individual's probability to secrete chemical defenses (Duchesne and Careau 2022).

The European fire salamander (*Salamandra salamandra*; hereafter referred to as "fire salamander") represents an interesting model system to study the integration of personality, warning coloration, and toxicity. Post-metamorphic salamanders inform potential predators about their potent toxins with their yellow-on-black coloration (Lüddecke et al. 2018; Caspers et al. 2020). However, high intraspecific variation in the dorsal proportion and pattern of yellow exists (Balogová and Uhrin 2015; Beukema et al. 2016; Seidel and Gerhardt 2016; Najbar et al. 2018; Preißler et al. 2019; Burgon et al. 2020; Barzaghi et al. 2022). This is surprising as a recent study showed that fire salamander models with higher dorsal proportions of yellow received less bite marks by predators (Caspers et al. 2020) and therefore, strong selection by predators should act on the yellow proportion. Furthermore, the dorsal proportion of yellow is heritable (Sanchez et al. 2019), enabling trait evolution if it is under selection. Fire salamanders have toxin glands across their entire back but the largest toxin glands are a pair of glands situated behind the eyes, called parotoid glands (Thiesmeier 2004; Lüddecke et al. 2018). The parotoid glands are mainly responsible for the production and storage of the toxins (Lüddecke et al. 2018). Therefore, the size of these glands (as has been shown in toads) correlates with the amount of toxic secretion produced and available for

122 defense (Toledo and Jared 1995; Phillips and Shine 2005; Mariotto et al. 2022). As the
123 production of both the toxins and the pigments responsible for the yellow patches are costly to
124 produce (Berenbaum 1995; Blennerhassett et al. 2019; Caspers et al. 2020; Barzaghi et al.
125 2022), it is surprising that no relationship between the proportion of yellow and the qualitative
126 toxicity (i.e., the chemical composition of the toxic secretion) exists (Vences et al. 2014;
127 Preißler et al. 2019; Sanchez et al. 2019; Burgon et al. 2020). However, besides the
128 composition of the secretion, the quantity of secretion (approximated by the parotoid gland
129 size) is an important characteristic of the chemical defenses of a fire salamander and could
130 show a correlation with coloration. Furthermore, the relationship between dorsal coloration
131 and personality has not been investigated yet (but see Aguilar et al. (2024) who found no
132 differences in movement behavior and space use between fire salamanders with red throats
133 and those with no red gular pigmentation). Still, personality in fire salamanders has been
134 investigated and confirmed before in studies with no focus on a relationship with coloration.
135 Krause and Caspers (2016) and Krause et al. (2021) found a statistical trend for a ($p = 0.08$)
136 positive correlation of the number of visited squares in an open-arena test of fire salamanders
137 at 27 months and 60 months of age. Furthermore, Chiocchio et al. (2024) showed significant
138 repeatability in seven behaviors related to activity, boldness, and exploration of both larval
139 and post-metamorphic juvenile fire salamanders. Therefore, repeatable behavioral differences
140 in fire salamanders exist but associations with coloration and/or toxicity have not been
141 investigated thoroughly, yet.

142 With our study, we aimed to close this gap, by investigating a potential relationship between
143 coloration, toxicity and personality traits. We used a standardized behavioral assay conducted
144 three times per individual with a husbandry period of 60 days between the first and second
145 assay with fire salamanders that were captured for another experiment (Schmidt et al. *in*
146 *preparation*). From video material collected during the behavioral assays, as well as during

147 the husbandry period, we investigated repeatable individual differences in activity,
148 exploration, and boldness. The aim of this study was two-fold. First, we wanted to examine
149 whether any behavioral trait showed a significant repeatability. Second we aimed to
150 investigate correlations between personality traits (i.e., behavioral syndromes (Sih et al.
151 2004)) and associations between the yellow proportion and relative parotoid gland size of an
152 individual with any given personality traits. As this study included males and females
153 collected over two seasons (spring and fall), we also investigated potential sex- and season-
154 specific differences in the behavioral traits or personality traits. As previously shown, we
155 expected fire salamanders to show significant repeatability in the different behaviors and
156 behavioral syndromes. We also expected correlations between personality traits and
157 coloration and gland size. Lastly, we expected sex- and season-specific differences in the
158 personality traits.

159 # Materials and methods

160 ## Collection of animals and study design

161 We collected 29 adult fire salamanders (snout-to-tail-length; STL > 130 mm) during two
162 rainy nights in April (spring) and one rainy night in September (fall) 2022 from three
163 locations (GPS-coordinates: 50.667, 7.083; 50.682, 7.118; and 50.687, 7.128) in a forest south
164 of Bonn, Germany (for exact sample sizes see Table S1). Immediately after encountering a
165 salamander, it was first photographed with a 2 € coin as size standard next to it (hereafter
166 referred as "Monitoring Photo") and then placed inside a box for the first behavioral assay
167 (see below). After the behavioral assay, the individual was sexed based on its body shape and
168 the form of the cloaca (Thiesmeier 2004; Seidel and Gerhardt 2016). Subsequently, the
169 salamander was transferred to a fauna box (Exo Terra, 37 cm × 22 cm × 16.5 cm, L×W×H).
170 The fauna boxes were filled with a layer of moist leaf litter and strips of dead tree bark
171 collected on-site. The animals were kept inside these boxes for a maximum of two days

before being introduced to standardized husbandry containers located within the building for animal behavior at Bielefeld University (Bielefeld, Germany). During the next 60 days, the salamanders were kept within the aforementioned husbandry containers (a detailed description of the husbandry conditions is provided in Schmidt et al. *in preparation*). The room the animals were maintained in was set at a 12:12 D:N-lightcycle with an air temperature of 14° C during the day and 8° C during the night. We ensured that all fire salamanders always had access to clean water and each animal was served earthworms (*Lumbricus terrestris*) once a week.

Behavioral experiments

Overall, each salamander was tested in three standardized behavioral assays. Each assay had the same protocol: at the start, the salamander was placed in the center of an opaque plastic box (KIS C Box S, 32.2 cm × 21.6 cm × 16.5 cm) that had been sprayed with tap water to ensure standardized levels of humidity within the boxes. The individual was recorded from above using a camera recorder (Sony, FDR-AX53) in "nightshot" mode that uses infrared light to avoid disturbance of the animals inside the boxes. Fire salamanders likely have a visual range between 300 and 700 nm (Sanchez et al. 2019; Aguilar et al. 2024). Therefore, infrared light defined as light with a wavelength of 780 to 1000 nm is expected to be not visible for fire salamanders. To minimize disturbance of the salamanders in the boxes, the boxes had opaque walls. For the first ten minutes, we scored different behavioral characteristics from the video recordings. First, we digitally placed a grid of 4 × 6 rectangles on top of the boxes, and similar to Krause and Caspers (2016) and Krause et al. (2021), we counted the number of grids visited at least once and the number of grid changes by an individual with the base of the tail as its "focal point". Second, within this first ten-minute timeframe, we noted the overall duration of all movements associated with locomotion (i.e., excluding head movements whereby the body does not change its location). Third, after the

first 10 minutes period, each individual was gently turned on its back for five times to simulate a predator attack (Baxter-Gilbert et al. 2021). The fire salamanders reacted to this intervention by immediately turning on their venters again, trying to flee, and by exuding toxins from their skin glands, indicating that this protocol was highly successful in simulating a dangerous situation for the salamanders. Indeed, in the study location, we have found dead fire salamanders with their bellies opened, likely by predatory birds or mammals who apply this same strategy to avoid the toxin glands on the dorsal side of the salamanders and consume the salamander's non-poisonous intestines from the ventral side (c.f. Toledo et al. (2010)). Following the predation simulation, the salamander was placed near the short wall of the box that is further way from the camera and a black plastic lid (Santos, D, 21.6 cm × 15 cm, L×W) that covered ~ 45 % of the box was placed on top of the box. This side then represented the "safe shelter" and the latency to leave the "shelter" within a period of ten minutes was noted. If an individual did not leave the "shelter" within the first ten minutes, it was assigned a maximum value of 600 seconds for that assay. This protocol is similar to the shelter-seeking and shelter-emergence tests used in Krause et al. (2011), Oswald et al. (2020), Hahn et al. (2023), and Chiocchio et al. (2024) but adds an additional risk-stimulus at the start to better differentiate between this behavioral trait and the traits measured in the first half of the assay (Réale et al. 2007; Kelleher et al. 2018).

The behavioral assays were conducted three times per individual. The first assay (hereafter referred to as "Field Test") was conducted in the field directly after capture of the salamander. The second assay (hereafter referred to as "Lab Test 1") was conducted at the end of the husbandry period (i.e., 60 days after the husbandry period started in the lab). The third test (hereafter referred as "Lab Test 2") was conducted three days after Lab Test 1 and few days before releasing the animals back to the wild. For the Lab Tests, the individuals were picked out of their husbandry boxes and placed in clean fauna boxes (see above), sprayed with tap

water. The salamanders were then transported outside of the facility were the assays continued as described above. Every assay was conducted well after dusk in complete darkness. After each assay, we placed the salamander on a grid paper and took another photo of it from above before returning the salamander to its husbandry box. After Lab Test 2, we returned the salamanders to their locations of collection in the forest. We assured that no salamander was infected with *Bsal* (*Batrachochytrium salamandrivorans*), before returning the salamanders.

During the 60 days husbandry period (details provided in Schmidt et al. *in preparation*), infrared cameras (Camera Security DVR System by Elro; DVR74S) were used to record each salamanders' activity. The 60 days were split into weekly blocks of behavioral observation or no observation, starting with one week of behavioral observation at the beginning of the husbandry period, followed by a week of no observation. At the end of the husbandry period, we had 30 days of video observation and we scored the salamander's surface activity (i.e., time visible and spent outside any hides and above the substrate). We summed the surface activity to 12 hours periods according to the day/night light cycle because of high zero inflation (i.e., the salamanders showed only very little surface activity). For each 12 hours period, we used a binary variable (yes/no) indicating whether an individual had been seen outside its hiding spots or not as the data was still highly zero inflated (85.6%). This approach is similar to the one applied by Chicchio et al. (2024) that scored the activity of post-metamorphic juvenile fire salamanders in their husbandry containers by noting events of movement. The behavioral assay videos and the husbandry videos were scored by different observers (MaMü and MS, respectively) who were not aware of the results of the respective other part of the experiment. Furthermore, the observers were blind to the identity of the individuals in the videos (please note that sexes can often be distinguished based on the body shape (see above) and because every individual has a unique color pattern, it is theoretically

possible to recognize an individual but this is unlikely given of the number of individuals and the quality of the videos).

Measurements of yellow proportion and relative parotoid gland size

For each individual, we had four dorsal photos (one before the Field Test, and another three after each behavioral assay). Of the four photos, we subjectively picked the one with the highest quality for the analysis of dorsal yellow proportion as the method is susceptible to issues such as shade or glare (Sanchez et al. 2018). In short, in each photo using the image manipulation software GIMP (https://www.gimp.org/), we cropped arms and legs and moved the remaining body of the salamander on a white background. The newly created image was then transferred to a python script that automatically quantifies the number of yellow and black pixels to calculate the yellow proportion (in %) in the image (Sanchez et al. 2018). The person responsible for this analysis (HJB) was not aware of the results of the behavioral assays and analysis of the husbandry period. For each of the four photos available for an individual, we measured the length of the parotoid gland on the left and the right side of the head as well as the STL in mm using the size standard provided in the photo using ImageJ (Abràmoff et al. 2004). As the parotoid glands have an elliptical shape (Toledo and Jared 1995; Thiesmeier 2004; Lüddecke et al. 2018), the length of the gland is a good approximation of the overall size of the gland (Bókony et al. 2019; Hudson et al. 2021).

Statistical analysis

All statistical analyses were conducted in the R statistical environment v. 4.4.0 (R Core Team 2022). Before any analyses we explored our data following (Zuur et al. 2010) to ensure our data included no unexplainable outliers or strongly confounded variables. To test for repeatability of the behavioral variables, we used the package “rptR” (Stoffel et al. 2017). For the behavioral variables from the behavioral assays, the formulae included the assay type (categorical: Field Test, Lab 1 Test, and Lab 2 Test), the air temperature at the start of the

assay (in °C) and the individual identity as a random intercept. Therefore, the repeatability estimate *R* provided by "rptR" represents the repeatability adjusted for potential sources of within-individual variation. As dependent variables, we used the aforementioned time spent moving (in seconds), the number of grids visited, the number of grid changes (with one added and thereafter $\log_{10}$-tranformed) as well as the latency to leave the shelter that was rank-transformed before analysis (Riley et al. 2017; Damas-Moreira et al. 2020; Mühlenhaupt et al. 2022). The transformations were conducted to ensure normality of the data. Therefore, we used the function *rptGaussian* with 1000 parametric bootstraps. For our fifth behavioral variable, visibility of the individuals during the husbandry period (binary), we used *rptBinary* with 1000 parametric bootstraps. The formula included the number of days since the start of the husbandry period as well as the light phase of the 12 hours period (categorical: diurnal (i.e., light turned on) and nocturnal (i.e., light turned off)) to adjust the *R*-values for these sources of within-individual variation. The formula also included a random intercept of individual identity as the grouping variable for which *R*-values were calculated. We also used linear mixed effects models (for the behavioral variables from the behavioral assays) as well as a generalized linear mixed effects model (for the visibility during the husbandry period variable) using the functions *lmer* and *glmer* provided by the package "lme4" (Bates et al. 2009). The models had the same formulae as described for *rptGaussian* and *rptBinary* for the specific variables to explore the role of the within-individual sources of variation on the behavioral variables. We visually confirmed the assumptions of model structure (normality of residuals, normality of random effects, linear relationship, homogeneity of variance, multicollinearity) using the function *check_model* provided in the package "performance" (Lüdecke et al. 2021) for the linear mixed effects models and by plotting the simulated residuals using the function *simulateResiduals* provided by the package "DHARMa" (Hartig 2022) for the generalized linear mixed effects model. The function *summary* provided by the package "lmerTest" (Kuznetsova et al. 2015) was used to identify any significant effects. To

conduct post-hoc multiple comparisons between the levels of the test type during the behavioral assays, we used the package "emmeans" (Lenth 2024) and the function *emmeans* therein.

If any behavioral variable showed a significant repeatability (based on a p-value < 0.05), we extracted and then simulated ($n_{simulations}$ = 1000) best linear unbiased predictors (BLUPs) using the function *sim* provided by the package "arm" (Gelman and Su 2007). Of the posterior distribution of BLUPs, we extracted the mean value as a "quantification of an individual's personality" for the given behavioral variable and use these simulated mean BLUP estimates in the subsequent analyses. The use of BLUPs in behavioral ecology has been criticized when uncertainty around the estimates is not carried forward in the analysis (Hadfield et al. 2010; Houslay and Wilson 2017). However, Dingemanse et al. (2020) showed that taking forward uncertainty in BLUP values resulted in biased over-conservative estimates while using mean BLUP values resulted in less precise, yet unbiased estimates (see Appendix S6 in Dingemanse et al. 2020). Therefore, we used the mean BLUPs from the posterior distribution for further analysis. If a behavioral variable did not show a significant repeatability, we added the fixed effects of season (categorical: fall, spring) and sex (categorical: female, male) to the mixed effects model to investigate the effects of season and sex on the trait.

Similar to the behavioral variables, we calculated relative parotoid gland size BLUPs by using a linear mixed effects model. As the dependent variable, the model included the $\log_{10}$-transformed parotoid gland length. As fixed effects, the model included the side (categorical: left and right), the timing of the photo (categorical: Monitoring Photo, After Field Test, After Lab 1 Test, After Lab 2 Test), and the $\log_{10}$-transformed STL taken from the same photo to account for the allometric relationship between STL and parotoid gland length (Bókony et al. 2019; Hudson et al. 2021). Both parotoid gland length and STL were $\log_{10}$-transformed prior to analysis, to ensure a linear relationship. As a random intercept, we included individual

identity. After confirming repeatability using *rptGaussian*, we simulated BLUPs using *sim* and used the mean BLUP value as an individual's indicator of its relative parotoid gland size.

To investigate the relationship of the behavioral BLUPs with each other (i.e., behavioral syndromes), we used Pearson's correlation coefficients provided by the function *ggpairs* in the package "GGally" (Schloerke et al. 2021). Lastly, to study the integration of personality traits with fire salamander coloration and toxicity, we used linear models with the function *lm* provided by base R (R Core Team 2022). As dependent variables, the models included the BLUP values for each behavioral variable that showed a significant repeatability. As explanatory effects, we used the relative parotoid gland size BLUP and the yellow proportion (divided by 100 to have the continuous variables on similar scales) of an individual. To test for the effects of season and sex on the personality of fire salamanders, we included both in the linear models. To test for a correlation between the relative parotoid gland size BLUP and the yellow proportion of fire salamanders, we calculated a Pearson's correlation coefficient using the function *cor.test* provided by base R (R Core Team 2022). All plots with the exception of the *ggpairs*-plot showing the correlations among the behavioral BLUPs, were created using "ggplot2" (Wickham 2011).

Results

Trait repeatability and the influence of within-individual sources of variation

Of the five behavioral variables (time spent moving, number of grids visited, number of grid changes, latency to leave the shelter, and likelihood to be outside in the husbandry box), all except the latency to leave the shelter were repeatable when adjusted for within-individual sources of variation (Table 1, Fig. 1). Therefore, the time spent moving, the number of grids visited, and the number of grid changes during the first part of the behavioral assay, as well as the likelihood to be outside of the hides and above the substrate in the husbandry box represent repeatable personality traits. In accordance with Réale et al. (2007) and Krause et al.

348 (2021) we refer to the personality trait represented by the number of grids visited as
349 Exploration hereafter. The personality traits represented by the time spent moving and the
350 number of grid changes we refer to as Activity 1 and Activity 2, respectively, hereafter.
351 Krause et al. (2021) also used the number of grid changes in their behavioral assay as a
352 representation of activity and we added the time spent moving (i.e., activity 1) as an
353 additional quantification of activity that is not necessarily related to exploration (Réale et al.
354 2007). We refer to the personality trait represented by the likelihood of emerging from hides
355 and the substrate within the husbandry box as Husbandry Activity hereafter as it also
356 represents the general level of activity (Réale et al. 2007; Kelleher et al. 2018).

357 **Table 1** The adjusted repeatability R_{adj} of each behavioral trait. Given are 95% confidence
358 intervals (*95% CI*) around the estimates as well as *p*-values calculated from likelihood ratio
359 tests. R_{adj} for the likelihood of emergence during husbandry is given on the link-scale.
360 Significant adjusted repeatabilities ($p < 0.05$) are presented in bold

Behavioral trait	R_{adj} (*95% CI*)	*p*
Time spent moving	**0.35 (0.11, 0.57)**	**< 0.01**
Number of grids explored	**0.29 (0.04, 0.51)**	**< 0.01**
Number of grid changes	**0.43 (0.18, 0.63)**	**< 0.01**
Latency to leave the shelter	0.03 (0, 0.24)	0.40
Likelihood of emergence during husbandry	**0.09 (0.09, 0.09)**	**< 0.05**

361

362 Every behavioral trait quantified in the behavioral assays with the exception of the latency to
363 leave the shelter was higher in the Field Test compared to the Lab 1 Test (Table 2, Table 3,
364 Fig. 1). In addition, the number of grids visited and the number of grid changes was lower in
365 the Lab 2 Test than in the Field Test. Moreover, the time spent moving and the number of grid
366 changes was lower in the Lab 1 Test compared to the Lab 2 Test. The latency to leave the

shelter was positively correlated with air temperature (Table 3). However, when including season and sex in the model, using the latency to leave the shelter as the dependent variable, this effect became non-significant (Table 3). Instead, in spring, fire salamanders showed a tendency to leave the hide later but this effect was marginally non-significant ($p = 0.056$, Table 3, Figure S1). During the husbandry period, individuals were more likely to be visible outside of hides and above the substrate in the nocturnal 12 hours phase. The likelihood of visibility in a given 12 hours phase also increased with the number of days since the start of the husbandry period (Table 4, Fig. 1E). Parotoid gland length was repeatable ($R_{adj} = 0.16$ (0.04, 0.29 CI), $p < 0.01$), strongly positively correlated with STL, and significantly larger in the Monitoring Photo compared to the other photos but there was no directed asymmetry in gland length (i.e., glands longer on one side than the other, Table S2, see Text S1 for a discussion of these results).

Table 2 Output from the linear mixed effects models examining differences in the time spent moving (s), the number of grids visited, and the number of grid changes (1 added and subsequently $\log_{10}$-transformed) during the first 10 minutes of the behavioral assays. Model estimates (β) of the fixed effects are presented with their corresponding standard errors (*SE*), and *t*-values. All significant effects ($p < 0.05$) are presented in bold. Test levels are given in parentheses following the variable name. Variance estimates (σ^2) are supplied for random effects and residuals. We also present post-hoc multiple comparisons of these three behavioral traits between all levels of Test and, in this case, p-values (p_{corr}) were corrected using a "Tukey" adjustment (Lenth 2024)

Model Summary					
Dependent variable	Model parameters	Model Output			
	Fixed effects	β	*SE*	*t*	*p*
Time spent moving	**Intercept (Field)**	**223.65**	**38.93**	**5.75**	**< 0.01**

	Test (Lab 1)	**-110.81**	**26.14**	**-4.24**	**< 0.01**
	Test (Lab 2)	-33.73	25.98	-1.30	0.20
	Temperature	-1.05	2.25	-0.47	0.64
	Random effects	σ^2			
	Individual ID	5155			
	Residual	9672			

Post-hoc multiple comparisons between the levels of Test for the time spent moving

Contrasts	*β*	*SE*	*t*	p_{corr}
Field – Lab 1	**110.8**	**26.1**	**4.24**	**< 0.01**
Field – Lab 2	33.7	26.0	1.30	0.40
Lab 1 – Lab 2	**-77.1**	**25.9**	**-2.98**	**0.01**

Model Summary

Dependent variable	Model parameters	Model Output			
	Fixed effects	*β*	*SE*	*t*	*p*
	Intercept (Field)	**10.62**	**1.89**	**5.61**	**< 0.01**
	Test (Lab 1)	**-6.47**	**1.29**	**-5.00**	**< 0.01**
	Test (Lab 2)	**-3.59**	**1.29**	**-2.79**	**< 0.01**
Number of grids visited	Temperature	0.08	0.11	0.75	0.46
	Random effects	σ^2			
	Individual ID	9.44			
	Residual	23.73			

Post-hoc multiple comparisons between the levels of Test for the number of grids visited

Contrasts	*β*	*SE*	*t*	p_{corr}
Field – Lab 1	**6.47**	**1.29**	**5.00**	**< 0.01**
Field – Lab 2	**3.59**	**1.29**	**2.79**	**0.02**

	Lab 1 – Lab 2	-2.89	1.28	-2.25	0.07

Model Summary

Dependent variable	Model parameters	Model Output			
	Fixed effects	*β*	*SE*	*t*	*p*
	Intercept (Field)	**0.98**	**0.15**	**6.69**	**< 0.01**
	Test (Lab 1)	**-0.50**	**0.10**	**-5.19**	**< 0.01**
	Test (Lab 2)	**-0.24**	**0.10**	**-2.49**	**0.02**
Number of grid changes	Temperature	0.01	0.01	1.32	0.19
	Random effects	σ^2			
	Individual ID	0.10			
	Residual	0.13			

Post-hoc multiple comparisons between the levels of Test for the number of grid changes

Contrasts	*β*	*SE*	*t*	p_{corr}
Field – Lab 1	**0.50**	**0.10**	**5.19**	**< 0.01**
Field – Lab 2	**0.24**	**0.10**	**2.49**	**0.04**
Lab 1 – Lab 2	**-0.26**	**0.10**	**-2.75**	**0.02**

Table 3 Output from the linear mixed effects models examining differences in the latency to leave the shelter during the second part of the behavioral assays. The latency was rank-transformed in order to achieve normality of residuals. Model estimates (β) of the fixed effects are presented with their corresponding standard errors (*SE*), and *t*-values. All significant effects ($p < 0.05$) are bold. Category levels are given in parentheses following the variable name. Variance estimates (σ^2) are supplied for random effects and residuals. We also present post-hoc multiple comparisons of these three behavioral traits between all levels of Test and, in this case, p-values (p_{corr}) were corrected using a "Tukey" adjustment (Lenth

2024). The first half of the table shows the summary and post-hoc multiple comparisons of the model that does not include the fixed effects of sex and season. The lower half of the table shows the summary and post-hoc multiple comparisons of the model that also includes sex and season effects

Model summary				
Model parameters	Model Output			
Fixed effects	*β*	*SE*	*t*	*p*
Intercept (Field)	**-0.84**	**0.31**	**-2.66**	**< 0.01**
Test (Lab 1)	0.45	0.23	1.97	0.05
Test (Lab 2)	0.29	0.23	1.29	0.20
Temperature	**0.04**	**0.02**	**2.25**	**0.03**
Random effects	σ^2			
Individual ID	0.02			
Residual	0.74			
Post-hoc multiple comparisons between the levels of Test				
Contrasts	*β*	*SE*	*t*	p_{corr}
Field – Lab 1	-0.45	0.23	-1.97	0.13
Field – Lab 2	-0.29	0.23	-1.29	0.41
Lab 1 – Lab 2	0.16	0.23	0.69	0.77
Model summary				
Model parameters	Model Output			
Fixed effects	*β*	*SE*	*t*	*p*
Intercept (Field, Female, Fall)	**-0.84**	**0.34**	**-2.47**	**0.02**
Test (Lab 1)	**0.48**	**0.24**	**2.04**	**0.046**
Test (Lab 2)	0.37	0.24	1.57	0.12

Temperature	0.03	0.02	1.23	0.23
Sex (Male)	-0.01	0.20	-0.03	0.98
Season (Spring)	0.42	0.21	1.99	0.056
Random effects	σ^2			
Individual ID	0.02			
Residual	0.74			

Post-hoc multiple comparisons between the levels of Test

Contrasts	*β*	*SE*	*t*	p_{corr}
Field – Lab 1	-0.48	0.24	-2.04	0.11
Field – Lab 2	-0.37	0.24	-1.57	0.27
Lab 1 – Lab 2	0.11	0.23	0.49	0.88

401

402 **Table 4** Output from the generalized linear mixed effects model examining differences in the
403 likelihood of an individual to be visible outside of any hides and above the substrate during
404 the 60 days husbandry period. Model estimates (β) of the fixed effects are presented with their
405 corresponding standard errors (*SE*) and are on the logit-scale. *z*-values are provided and all
406 significant effects ($p < 0.05$) are presented in bold. Phase levels are given in parentheses
407 following the variable name. Variance estimates (σ^2) are supplied for the random effect of
408 individual identity (random intercept)

Model summary

Model parameters	Model Output			
Fixed effects	*β*	*SE*	*z*	*p*
Intercept (Diurnal)	**-3.65**	**0.27**	**-13.34**	**< 0.01**
Days since start	**0.03**	**0.00**	**5.76**	**< 0.01**

Phase (Nocturnal)	**1.35**	**0.17**	**8.15**	**< 0.01**
Random effect	σ^2			
Individual ID	0.79			

409

410 Behavioral syndromes and the integration of personality, proportion of yellow, and
411 relative parotoid gland size

412 All personality traits expressed in the behavioral assays (i.e., Activity 1: time spent moving,
413 Exploration: number of grids visited, and Activity 2: number of grid changes) showed strong
414 positive correlations with each other, while none of these personality traits correlated with the
415 Husbandry Activity personality trait (Fig. 2). The proportion of dorsal yellow coloration was
416 not correlated with the relative size of the parotoid gland (Figure S2). Neither, Activity 1,
417 Exploration, nor Activity 2 showed correlations with the proportion of dorsal yellow
418 coloration or the relative size of the parotoid gland (Table 5). All personality traits were
419 expressed more strongly by fire salamanders collected in fall compared to spring (Table 5,
420 Figure 3, Figure 4). Husbandry Activity was positively correlated with relative parotoid gland
421 size but not with the proportion of dorsal yellow coloration (Table 5, Figure 4). No other
422 differences in the personality traits were found (Table 5, Figure 3).

423 **Table 5** Output of the linear models examining differences in the personality traits. Model
424 estimates (β) are presented with their corresponding standard errors (*SE*). *t*-values are
425 provided and all significant effects ($p < 0.05$) are presented in bold. The levels of sex and
426 season are given in parentheses following the variable names. The proportion of yellow (%)
427 was divided by 100 prior to analysis. Two individuals were removed from this analysis as
428 their sex was ambiguous (see Table S1)

Model Summary

Dependent variable	Model parameters	Model Output			
		β	*SE*	*t*	*p*
Activity 1 (BLUP)	Intercept (Female, Fall)	-7.55	52.13	-0.15	0.89
	Proportion of yellow	44.71	178.54	0.25	0.81
	Relative parotoid gland size	371.11	1023.32	0.36	0.72
	Sex (Male)	30.05	28.19	1.07	0.30
	Season (Spring)	**-46.00**	**21.96**	**-2.09**	**0.048**
Exploration (BLUP)	Intercept (Female, Fall)	0.35	1.87	0.19	0.85
	Proportion of yellow	0.11	6.41	0.02	0.99
	Relative parotoid gland size	-9.52	36.75	-0.26	0.80
	Sex (Male)	1.62	1.01	1.60	0.12
	Season (Spring)	**-2.42**	**0.79**	**-3.07**	**< 0.01**
Activity 2 (BLUP)	Intercept (Female, Fall)	-0.08	0.23	-0.35	0.73
	Proportion of yellow	0.55	0.79	0.69	0.50
	Relative parotoid gland size	-1.70	4.52	-0.38	0.71
	Sex (Male)	0.09	0.12	0.72	0.48
	Season (Spring)	**-0.28**	**0.10**	**-2.86**	**< 0.01**
Husbandry Activity (BLUP)	Intercept (Female, Fall)	0.39	0.51	0.75	0.46
	Proportion of yellow	1.00	1.76	0.57	0.57
	Relative parotoid gland size	**23.31**	**10.08**	**2.31**	**0.03**
	Sex (Male)	-0.41	0.28	-1.46	0.16
	Season (Spring)	**-0.89**	**0.22**	**-4.12**	**< 0.01**

429

Discussion

Fire salamanders show high inter-individual differences in their dorsal coloration. Here, we tested whether these individual differences in coloration might correlate with personality traits and parotoid gland size. We found strong evidence for personality in fire salamanders as four behaviors related to activity and exploration were repeatable. Only one behavior, the latency to leave a shelter after a simulated predator attack that is related to "boldness" was not repeatable. We found only little evidence for the integration of personality with the dorsal proportion of yellow and the relative size of the parotoid glands as all these traits are relevant in an anti-predator context. Only the activity shown by a fire salamander in the husbandry box was positively correlated with the relative parotoid gland size. All personality traits expressed by an individual in the behavioral assay showed strong correlations and thereby, represent a behavioral syndrome, but we found no correlations of any personality trait with the activity in the husbandry box. Interestingly, the fire salamanders showed more activity and exploration when collected in fall compared to spring, indicating seasonal effects on these personality traits.

The timing of the behavioral assay (i.e., Field, Lab 1 or Lab 2) had a strong effect on fire salamander behavior, as the salamanders moved more and explored more when tested in the field directly after collection than during the tests conducted after the husbandry period. One likely driver of this variation might be the internal motivation of the salamanders. Fire salamanders captured for this experiment in the field were outside their hides. In contrast, most fire salamanders were hidden under their hides or under the moss in the husbandry containers before being tested in the Lab 1 and Lab 2 test. We standardized humidity levels in the boxes by spraying the insides with water and all assays were conducted well after dusk in complete darkness. Therefore, an influence of other abiotic factors that might have been different between the field and lab assays can be ruled out. Similarly, temperature did not

affect the time spent moving, the number of grids visited or the number of grid changes in the box. Future studies of wild-caught salamanders or potentially even other animals that are tested in the lab and maintained in the lab should consider these results as they indicate that findings from tests conducted in the lab might not be fully transferable to the natural behavior of the animal.

Only latency to leave the shelter after a simulated predator attack, a behavior related to boldness was not significantly repeatable and was not affected by the timing of the assay. As this experimental approach was used on fire salamanders for the first time (but see Baxter-Gilbert et al. (2021) for a similar protocol for toads), it is difficult to make meaningful comparisons. However, we argue that this experimental approach allows for better separation between behaviors along the personality axes proposed by Réale et al. (2007) as an additional risk stimulus is applied and the fire salamanders already had some time to explore the box in the previous ten minutes. This assumption is also supported by the inconsistency in repeatabilities comparing the latency to leave the shelter with the other three behaviors observed during the behavioral assays (i.e., time spent moving, number of grids visited, and number of grid changes) that occurred directly before the simulated predator attack. Therefore, our results support the assumption that the behavioral trait indicated by the latency to leave the shelter is different from the behavioral trait(s) indicated by these other three behavioral characteristics. The latency to leave the shelter might be more strongly affected by the internal motivation of an individual and/or external factors, such as air pressure that we could not control for, explaining why this trait was not repeatable.

Similarly, internal motivation (e.g., to forage) likely also caused the positive effect of number of days in husbandry on the likelihood of the fire salamanders to emerge in the husbandry box. Food, water and a wet box were provided (for details see Schmidt et al. *in preparation*) in the husbandry box but to access these, fire salamander had to emerge from their hides or

480 from underneath the substrate. As fire salamanders are mostly nocturnal in our study area
481 (*personal observation*), it is not surprising that the salamanders were more likely to be visible
482 in the husbandry box in the nocturnal phase. Still, on some occasions, fire salamanders
483 emerged during the diurnal phase. These are exciting findings that are discussed in more
484 detail in Schmidt et al. (*in preparation*).

485 Four of the five behaviors observed in this experiment showed low to moderate repeatability
486 values (cf. Bell et al. (2009)). These findings are in line with previous research by Chiocchio
487 et al. (2024) who found significant repeatability of eight of nine behaviors tested in larval and
488 juvenile (post-metamorphic) fire salamanders. Only the sheltering behavior in a novel
489 environment that could be related to the latency to leave the shelter in our behavioral assays
490 (but see above for methodological differences) was not repeatable. While amphibians
491 represent a vertebrate group that has received less attention when studying animal personality
492 (Kelleher et al. 2018), we now have consistent evidence of repeatable behavioral differences
493 in fire salamanders. Surprisingly, covariation with the dorsal proportion of yellow did not
494 explain these consistent behavioral differences. Furthermore, the relative size of the parotoid
495 glands could only partially explain our findings. While the general literature supports weak
496 but significant covariation between "state" variables such as hormone levels or body size with
497 personality traits related to aggressiveness, boldness, exploration, and activity (Niemelä and
498 Dingemanse 2018), our study included other "state" variables (i.e., coloration and relative
499 parotoid gland size). One explanation for the lack of covariation might be that we simply
500 included the "wrong" variables in this study. The dorsal proportion of yellow of a post-
501 metamorphic fire salamander is strongly influenced by the larval environment (Pederzoli et al.
502 2003; Krause and Caspers 2016; Sanchez et al. 2019; Barzaghi et al. 2022) and changes
503 gradually over the long lifespan of a fire salamander (Balogova et al. 2016; Krause et al.
504 2021; Barzaghi et al. 2022). Therefore, multiple drivers of color variation are already known

505 for fire salamanders. Even small proportions of yellow coloration on the dorsum might be
506 sufficient to provide an effective defense from potential predators alleviating the importance
507 of additional behavioral adaptations. This assumption is supported by studies that show no
508 correlation between the composition of the toxic secretion (i.e., qualitative toxicity) and the
509 proportion of yellow in fire salamanders (Preißler et al. 2019; Sanchez et al. 2019; Burgon et
510 al. 2020) as well as our finding that the dorsal proportion of yellow did not correlate with the
511 relative size of the parotoid glands (i.e., quantitative toxicity). Furthermore, the yellow
512 proportion might also play a role in a mate choice context as males usually have a higher
513 dorsal proportion of yellow than females (Balogová and Uhrin 2015; Preißler et al. 2019;
514 Mühlenhaupt et al. 2025), indicating the importance of other selective pressures than
515 predation. Although we found no correlation between the aposematic coloration and
516 personality traits in this specific study, we argue that studying the covariation of traits related
517 to aposematism and the personality of animals remains an exciting new research field in
518 animal behavior that could help us better understand the paradoxically high variation in
519 coloration in many aposematic species.

520 We found a significant positive correlation between the personality trait expressed as activity
521 during the husbandry period and the relative size of the parotoid glands of a fire salamander.
522 Fire salamanders with larger parotoid glands, i.e. those that are better protected against
523 potential predators, also showed higher activity during the 60 days of husbandry. This is in
524 contrast with a previous study that found no correlation between activity and parotoid gland
525 size in natterjack toads (*E. calamita*) (Zamora-Camacho 2022). However, the repeatability of
526 this behavior was not estimated in the study and the experimental set-up resembled the set-up
527 of our behavioral assays more than the husbandry set-up, making the contrasting results
528 difficult to interpret. Fire salamanders with larger parotoid glands are better protected. Thus,
529 can be more active to find more mates and food sources and can explore more. However,

since chemical defenses are energetically costly (Blennerhassett et al. 2019), the fire salamanders with larger parotoid glands in relation to body size might have compensated for this increased energetic cost by spending more time foraging in the husbandry boxes (Smith and Blumstein 2008). Future studies should quantify the energetic costs of toxicity in fire salamanders. During observation of Fig. 4, we noticed a potential sex-specific difference in the association of activity during the husbandry period and the relative size of the parotoid glands. As we did not initially plan to consider any interactions in our models given our low sample size, we present these exploratory, post hoc results in the Supplementary Materials, and discuss them with appropriate caution in their interpretation (Figure S3, Table S3, Text S2).

All personality traits were expressed higher in fire salamanders collected in fall compared to fire salamander collected in spring (the latency to leave the shelter tended to be higher in spring, see Table 3). This is in line with the general consensus that the main activity period of fire salamanders is between September and October (Thiesmeier 2004; Dehling 2024). It is believed that in fall, fire salamanders mainly emerge from their hides in order to search for mating opportunities, to forage, and to migrate to their hibernation quarters (Thiesmeier 2004). Our study is the first to show that wild-caught fire salamanders kept in husbandry also show higher activity and exploration in fall compared to spring indicating the strong effect of seasonal changes on fire salamander behavior. It would be very interesting to test the same individual in different seasons to estimate the proportion of variation in behavior that is explained by season in wild fire salamanders. This would require a long-term behavioral experiment with high sample sizes as recapture rates can be quite low for adult fire salamanders (Burgstaller et al. 2021; Kiss et al. 2022). At the current state, our study provides evidence for short-term personality that is strongly affected by season, however, Krause and Caspers (2016) and Krause et al. (2021) found a statistical trend ($p = 0.08$) of a positive

555 correlation of the number of visited squares in an open field test in fire salamanders tested
556 almost three years apart. Therefore, long-term repeatability of behavioral traits in fire
557 salamanders is quite conceivable.

558 In this study, we find strong correlations among three personality traits that were expressed
559 within the behavioral assays. In contrast, no personality trait correlated with the Husbandry
560 Activity personality trait expressed during the husbandry period. As Husbandry Activity was
561 a measure of activity over a 60 days period, we might question whether the behavioral assays
562 we used are suitable to study activity. The behavioral syndrome represented by the three
563 metrics (i.e., time spent moving, number of grids visited, and number of grid changes) likely
564 better fits the framework of exploration as it is a behavior expressed in a novel environment
565 while the Husbandry Activity personality trait better fits the framework of activity (i.e.,
566 general level of activity in a familiar environment, cf. Réale et al. (2007)). However, previous
567 studies have used similar approaches to ours, even for fire salamanders. For example, Krause
568 and Caspers (2016) and Krause et al. (2021) also distinguished between the number of grids
569 visited and the number of grid changes by fire salamanders in an open field test but did not
570 report correlations among these two variables. Interestingly, Chiocchio et al. (2024) found
571 correlations among personality traits, even when these traits were expressed in familiar versus
572 novel environments. Ultimately, the lack of a statistically significant correlation between the
573 personality traits in the behavioral assay and the Husbandry Activity personality trait in our
574 study might also be the results of a low sample size in our study making it difficult for
575 statistical models to find a significant effect, even when that effect was true. Nonetheless,
576 future studies should carefully plan their behavioral tests in order to avoid quantifying the
577 same trait using different metrics (Réale et al. 2007; Kelleher et al. 2018) and the results of
578 our study provide a good basis for further research.

Conclusions

Our study highlights the prevalence of animal personality in wild fire salamanders. While there was only weak support for the integration of personality traits with the relative toxin gland size of a fire salamander and no correlation of any personality trait with coloration, we found a strong effect of season on the expression of a given personality trait and a correlation of toxin gland size with the general activity level of an individual. Throughout the discussion, we make suggestions for future avenues of research, highlight potential methodological pitfalls, and encourage further research on the causes and consequences of consistent behavioral differences in aposematic animals.

Supplementary Information

Supplementary information are provided in this file.

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Author contribution

MaMü and BAC conceived the idea and designed the study. MaMü, HJB, MS, and LS conducted the fieldwork and experiments. MaMü, HJB, and MS analyzed the data. MaMü and MaMo conducted the statistical analysis. MaMü, MaMo, and BAC interpreted the results and conceived the first draft of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data availability

All data and code will be made available online at the Open Science Framework (OSF) upon acceptance of this manuscript for publication.

Declarations

Ethics approval

All methods of this study have been approved by the LANUV: Az 81-02.04. 2021.A437. The collection of wild fire salamanders from the Kottenforst near Bonn was authorised by the City of Bonn. After the experiment, all fire salamanders have been released at the site of capture.

Competing interests

The authors declare no competing interests.

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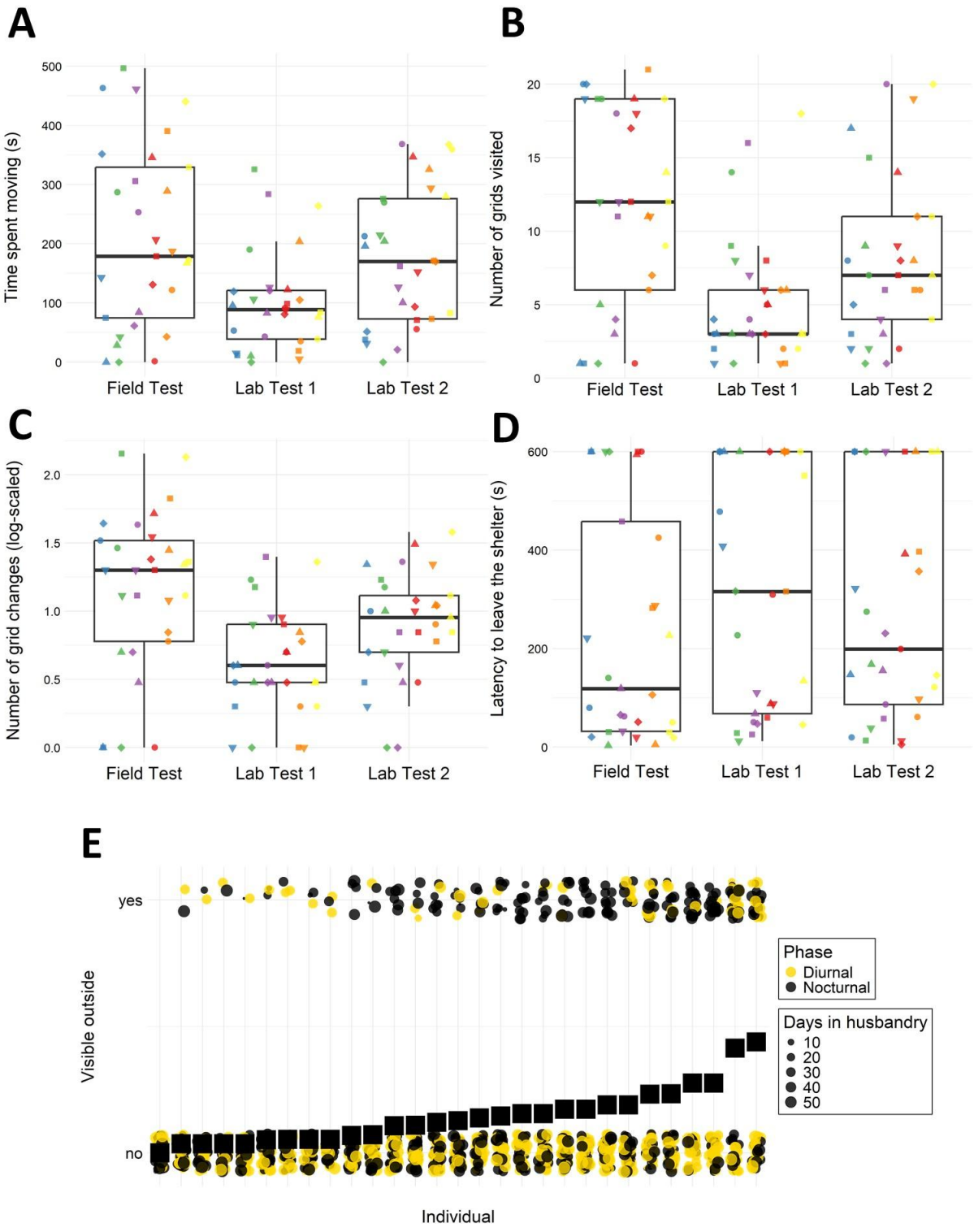
814 Figures



815

816 **Fig. 1A** The time spent moving, **B** the number of grids visited, **C** the number of grid changes

817 (on a $\log_{10}$-scale), and **D** the latency to leave the shelter during the second part of the

818 behavioral assay of a fire salamander during each of the three tests of the behavioral assays.

819 Given are boxplots with the thick bar representing the median, the box representing the 2nd
820 and 3rd quartile, and the whiskers representing data outside these quartiles. Each symbol has a
821 unique combination of color and shape and represents the value of a specific individual for the
822 given trait in a given test. **E** The mean likelihood to be visible in the husbandry box (black
823 squares) for each individual. The dots represent a specific 12 hours period during the
824 husbandry experiment colored by phase (yellow – diurnal; black – nocturnal) and the size of
825 the dot represents the days that have passed since the start of the husbandry experiment for a
826 given period (large dots represent periods that occurred later during the experiment). If the
827 fire salamander was visible in the given period, the dot is placed on the top and if it was not
828 visible, the dot is placed on the bottom of the graph

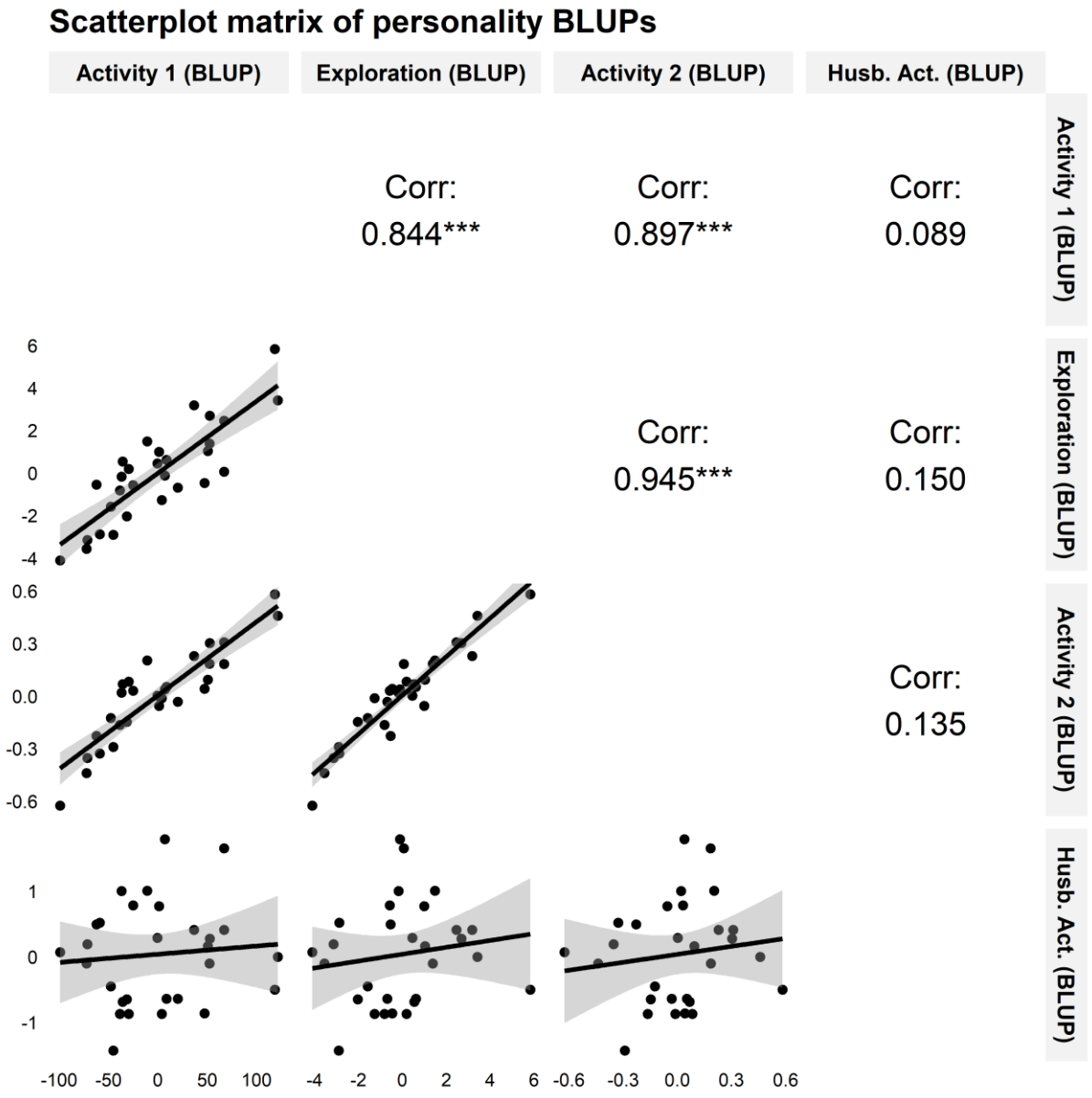
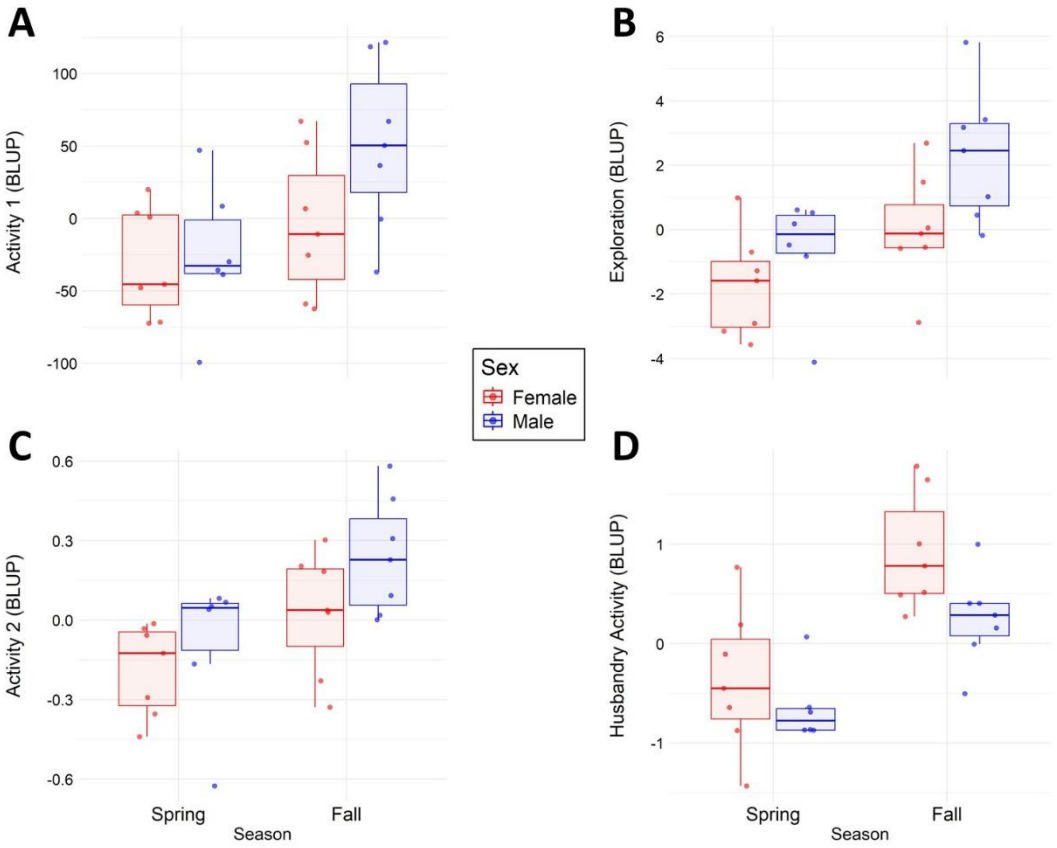
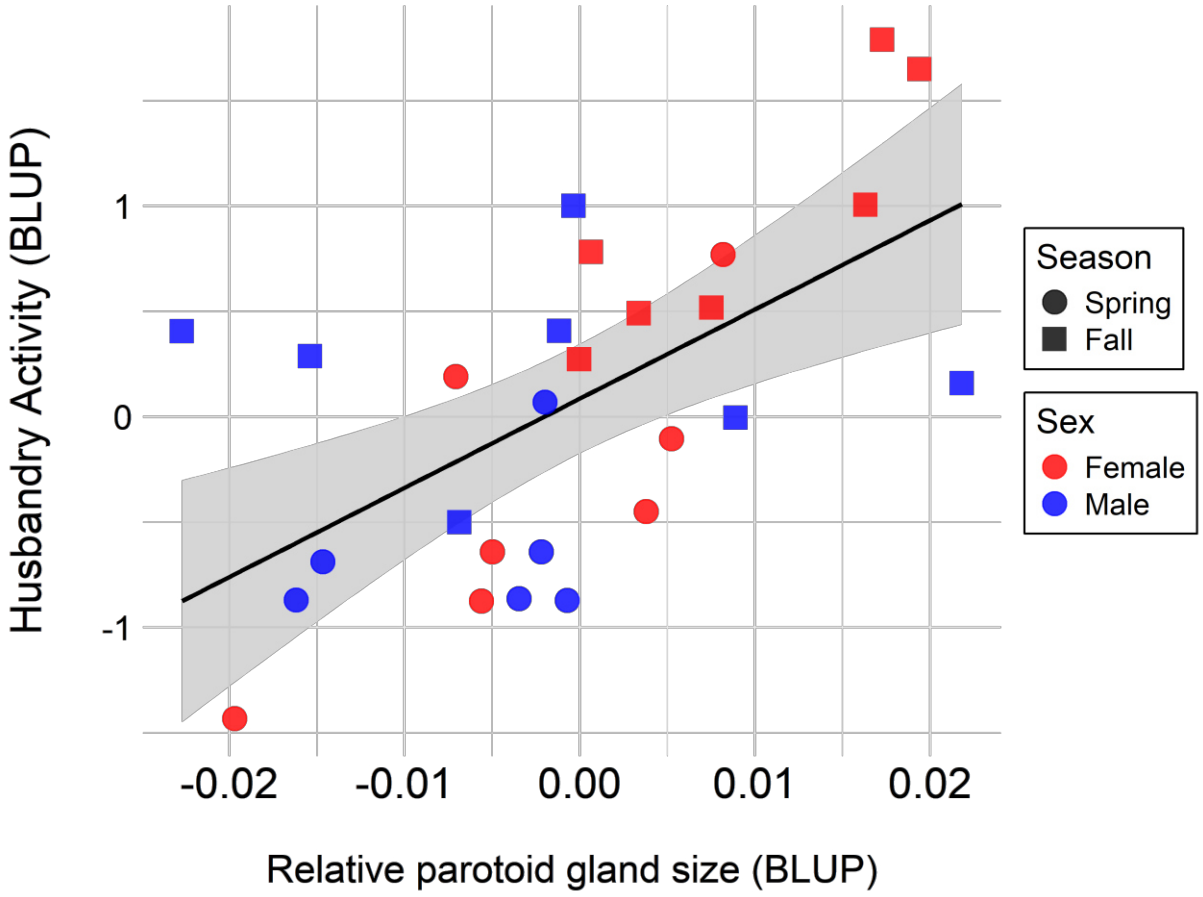


Fig. 2 The correlations (i.e., behavioral syndromes) between the personality traits. The upper right correlation coefficients (Corr.) were calculated from Pearson's correlation tests and the asterisks provide information on the *p*-value of the correlation (* < 0.05, ** < 0.01, *** < 0.001). The lower left plots show the relationships between the personality traits with black linear regression lines and corresponding 95% confidence intervals (in grey) provided for visualization



838

839 **Fig. 3A** Activity 1, **B** Exploration, **C** Activity 2, and **D** Husbandry Activity personality traits
840 of fire salamanders. Boxplots by season (left – spring, right – fall) and sex (red – females,
841 blue – males) are shown whereby the thick line represents the median, the box shows the 2nd
842 and 3rd quartile and the whiskers show the distribution outside the 2nd and 3rd quartile. Dots
843 depict the specific values for each individual. Two individuals were omitted from the plots
844 due to ambiguity of sex (see Table S1)



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846 **Fig. 4** The relationship between Husbandry Activity and relative parotoid gland size shown
847 using a linear regression line with a 95% confidence interval (shaded in grey). The dots
848 represent the individual Best Linear Unbiased Predictors (BLUPs) and are colored by sex (red
849 – female, blue – male). Circles represent fire salamanders collected in spring and squares
850 represent fire salamanders collected in fall. Two individuals were omitted from the plots due
851 to ambiguity of sex (see Table S1)

852

Behavioral Ecology and Sociobiology

The bright, the bold and the toxic: do coloration, personality, and toxicity represent an integrated phenotype in fire salamanders?

SUPPLEMENTARY INFORMATION

Max Mühlenhaupt[1]*, Henry J. Bohny[1], Maria Moiron[2,3], Manuela Schmidt[1], Laura Schulte[1], Barbara A. Caspers[1,3]

[1] Department of Behavioural Ecology, Bielefeld University, Konsequenz 45, 33615 Bielefeld, Germany

[2] Department of Evolutionary Biology, Bielefeld University, Konsequenz 45, 33615 Bielefeld, Germany

[3] Joint Institute for Individualisation in a Changing Environment (JICE), Bielefeld University and University of Münster, Konsequenz 45, 33615 Bielefeld, Germany

* Corresponding author, E-mail address: max.muehlenhaupt@uni-bielefeld.de

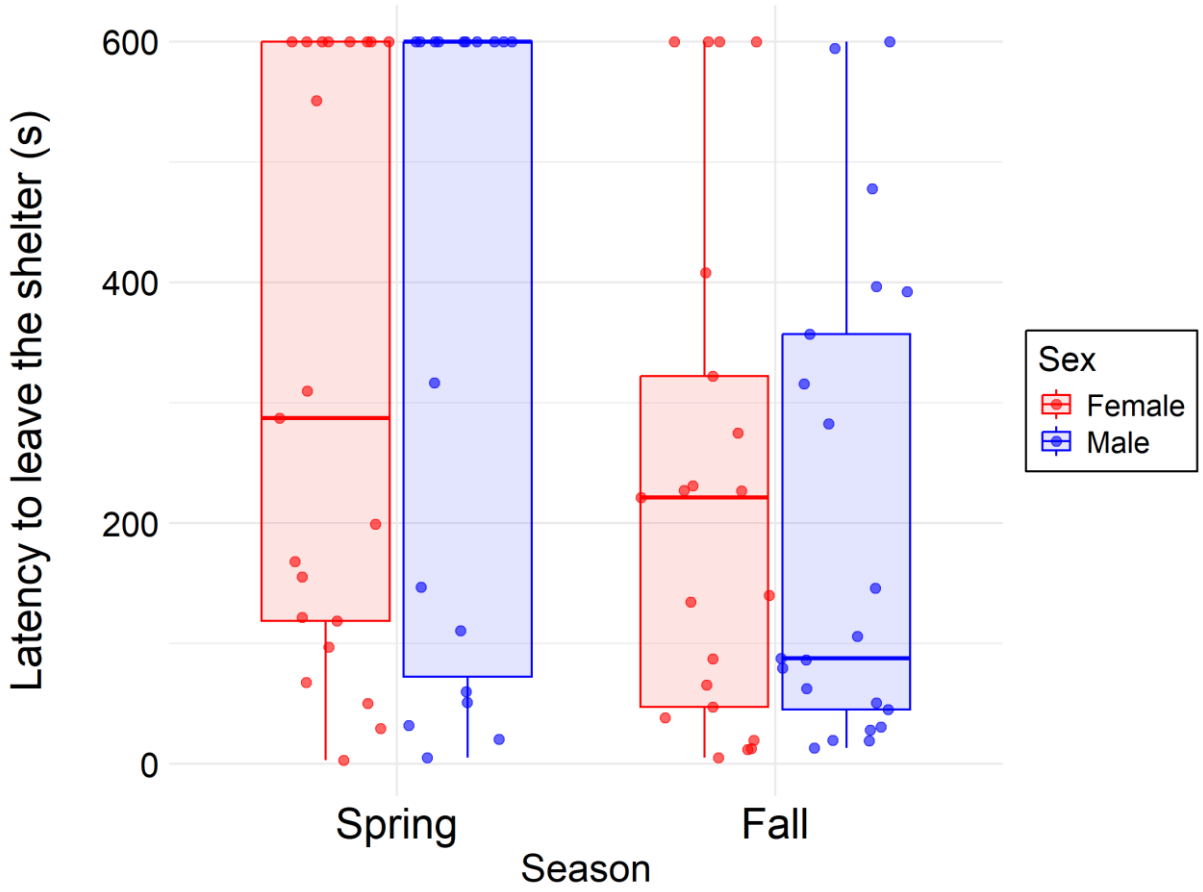

Figure S1 Boxplots with sample points that show the latency to leave the shelter during the second part of the behavioral assay colored by sex (red – female; blue – male) and ordered by season (left – spring; right – fall). As Test type did not have an effect on the latency and this trait was not repeatable (Table 1 and Table 3), the data were pooled. The thick bars represent the median, the box represents the 2nd and 3rd quartile, and the whiskers represent data outside these quartiles

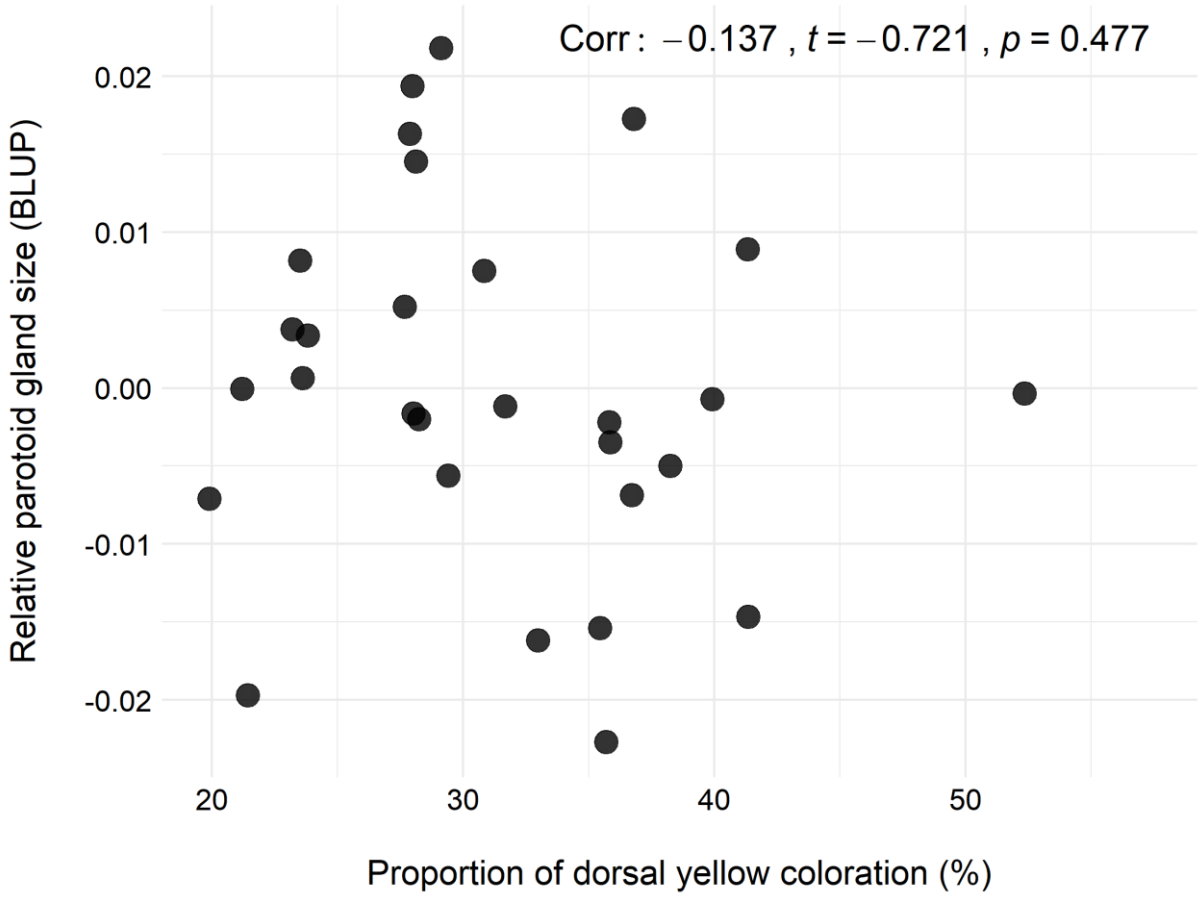

Figure S2 Scatterplot of the relative parotoid gland size BLUP on the y-axis and the proportion of dorsal yellow coloration (%) on the x-axis. The correlation of both variables was not significant ($p = 0.48$). Correlation coefficient (Corr), t-value, and p-value calculated from a Pearson's correlation test are provided in the top right corner

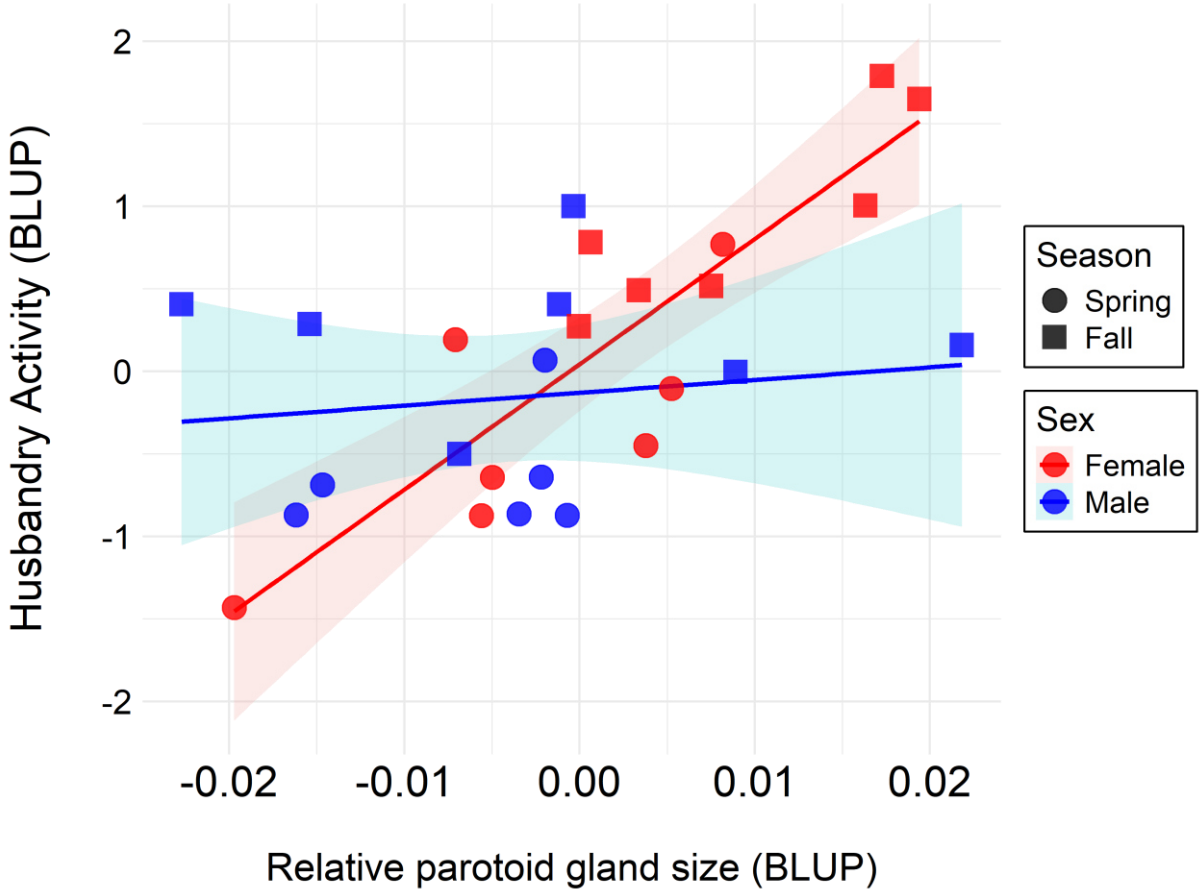

Figure S3 The relationship of the Husbandry Activity personality trait with the relative parotoid gland size. Dots are colored by sex (red – female, blue – male). Circles represent fire salamanders collected in spring and squares represent fire salamanders collected in fall. Two regression lines (red – female, blue – male) are presented with their corresponding 95 % confidence intervals in the respective colors. Two individuals were removed from this analysis as their sex was ambiguous (see Table S1)

Table S1 Information on the fire salamanders collected for this study. During two rainy nights in spring, we collected 14 individuals and during one rainy night in autumn, we collected 15 individuals. Sex was determined based on morphological characteristics (body shape and shape of cloacal region; e.g., Thiesmeier (2004); Seidel and Gerhardt (2016)). However, for

two individuals (one in spring and one in autumn, respectively), the sex determination was ambiguous. Therefore, these two individuals were coded as "NA" for our statistical analyses

Date	Sex: Female	Sex: Male	Sex: Unknown
06.04.2022	3	1	-
24.04.2022	4	5	1
13.09.2022	7	7	1

Table S2 Output from the linear mixed effects model examining differences in the parotoid gland length (log_{10}-tranformed) by side and different photos of each individual taken during the experiment. Model estimates (β) of the fixed effects are presented with their corresponding standard errors (*SE*), and *t*-values. All significant effects ($p < 0.05$) are marked in bold. Photo and Side levels are given in parentheses following the variable name. Snout-to-tail-length (STL) was also log_{10}-transformed. All model estimates are on the log_{10}-scale. Variance estimates (σ^2) are supplied for random effects and residuals. We also present post-hoc multiple comparisons all levels of Photo and, in this case, p-values (p_{corr}) were corrected using a "Tukey" adjustment (Lenth 2024)

Model summary				
Model parameters	Model Output			
Fixed effects	β	*SE*	*t*	*p*
Intercept (Left, Field)	-0.25	0.16	-1.63	0.11
STL	**0.63**	**0.07**	**8.96**	**< 0.01**
Side (Right)	-0.01	0.00	-1.32	0.19
Photo (Lab 1)	-0.01	0.01	-1.33	0.19
Photo (Lab 2)	**-0.02**	**0.01**	**-2.42**	**0.02**

Photo (Monitoring)	**0.02**	**0.01**	**3.15**	**< 0.01**
Random effects	σ^2			
Individual ID	0.00			
Residual	0.00			

Post-hoc multiple comparisons between the levels of Photo

Contrasts	*β*	*SE*	*t*	p_{corr}
Field – Lab 1	0.01	0.01	1.33	0.55
Field – Lab 2	0.02	0.01	2.42	0.08
Field – Monitoring	**-0.02**	**0.01**	**-3.15**	**0.01**
Lab 1 – Lab 2	0.01	0.01	1.10	0.69
Lab 1 – Monitoring	**-0.03**	**0.01**	**-4.53**	**< 0.01**
Lab 2 – Monitoring	**-0.04**	**0.01**	**-5.63**	**< 0.01**

Table S3 Output of the linear model examining differences in the Husbandry Activity personality trait. Model estimates (β) are presented with their corresponding standard errors (*SE*). *t*-values are provided and all significant effects ($p < 0.05$) are presented in bold. The levels of sex and season are given in parentheses following the variable names. The proportion of yellow (%) was divided by 100 prior to analysis. Two individuals were removed from this analysis as their sex was ambiguous (see Table S1)

Model summary

Model parameters	Model Output			
Fixed effects	*β*	*SE*	*t*	*p*
Intercept (Female, Fall)	0.49	0.43	1.14	0.27
Proportion of yellow	0.00	1.50	0.00	1.00

Relative parotoid gland size	**54.11**	**12.68**	**4.27**	**< 0.01**
Sex (Male)	-0.30	0.23	-1.28	0.22
Season (Spring)	**-0.76**	**0.19**	**-4.10**	**< 0.01**
Relative parotoid gland size : Sex (Male)	**-52.85**	**16.27**	**-3.25**	**< 0.01**

70

71 **Text S1. The influence of snout-to-tail-length, directed asymmetry, and photo type on**
72 **the parotoid gland length of fire salamanders**

73 Our linear mixed effects model investigating the effects of snout-to-tail-length (STL), the side
74 (left or ride), and the photo type (Monitoring, Field, Lab 1, Lab 2) on the parotoid gland
75 length as well as individual variation in the parotoid gland length when controlled for the
76 aforementioned effects showed a strong positive correlation with STL, that glands were
77 longer when measured from the Monitoring photo but showed no directed asymmetry (i.e.,
78 consistent differences between sides, Table S2). Furthermore, when adjusting for these fixed
79 effects, the relative parotoid gland length was repeatable for an individual as confirmed with
80 *rptGaussian* in the package rptR (Stoffel et al. (2017), R_{adj} = 0.16 (0.04, 0.29 CI), $p < 0.01$). It
81 is unsurprising that parotoid gland length and STL are highly positively correlated as the
82 gland tissue grows with the overall body size of a fire salamander (Toledo and Jared 1995).
83 Including STL in the model and extracting the mean BLUPs from this model with the arm R-
84 package (Gelman and Su 2007) therefore provides an estimate of an individual's parotoid
85 gland length relative to STL which indicates if an individual showed more or less investment
86 into the parotoid gland tissue compared to the mean. As the parotoid gland tissue is costly to
87 produce and maintain (Blennerhassett et al. 2019), an individual with a larger relative parotoid
88 gland size BLUP invested proportionately more into this defensive tissue. However, in the
89 future, the implications of overall larger parotoid glands should also be considered as it is the

absolute gland tissue size and not the size of the gland relative to the body size that dictates how much toxin is exuded by an individual (e.g., Phillips and (2005)). For example, the overall larger parotoid glands of adult fire salamanders are likely one reason why adults experience less predation pressure compared to smaller sub-adult fire salamanders that have smaller parotoid glands (Thiesmeier 1990, 2004).

Interestingly, the side had no effect on the length of the parotoid gland (Table S2), indicating that there is no directed asymmetry in the parotoid gland length of fire salamanders. Directed asymmetry would be expected if fire salamanders for example have a preferred side that they expose to attackers in order to present the attacker with the strongest chemical defense on that side. However, many predators of fire salamanders are birds such as owls (Strigiformes, Thiesmeier (2004)) that strike from above rather than from the side. Furthermore predators such as snake (e.g., *Natrix natrix*) attempt to swallow the salamander whole with the tail first (*personal observations*). Therefore, a directed asymmetry in parotoid gland size might not provide a selective advantage. instead, future studies could investigate the role of fluctuating asymmetry in gland length (i.e., non-consistent differences in left and right gland lengths between individuals and the strength of these differences) as fluctuating asymmetry could be an important indicator of developmental stress caused by e.g., environmental pollution (Wright and Zamudio 2002; Graham et al. 2010; Alarcón-Ríos et al. 2024).

We found that parotoid glands were longer when measured from the Monitoring photo compared to the other three photos available for each individual (Table S2). This is likely a result of the different size standards used and represents a methodological bias. In the Monitoring photo, we used a 2 € coin, whereas in all other photos, we used a gridded paper. It is easier to set the standard using a visible straight line (i.e., the grids on the piece of paper) compared to trying to draw a line of the diameter of a 2 € coin. This could have caused this systematic bias. Luckily, we have four photos available per individual and were able to

control for this bias. These results, however, highlight the importance of methodological consistency.

Text S2. Sex-specific differences in the relationship between Husbandry Activity and the relative parotoid gland length

Including an interaction of sex and relative parotoid gland size in the model investigating differences in Husbandry Activity, indicated that Husbandry Activity was positively associated with relative parotoid gland length in females only but not in males (Figure S3, Table S3). While this sex-specific association is very interesting, we did not initially include an interaction in the model given the low sample size. Post hoc, we included this interaction after observation of Fig. 4 but discuss this finding here and not in the main manuscript due to the limitations of our sample size. Such a sex-specific association indicates that only females show higher Husbandry Activity when they display larger parotoid glands while in males, there is no association between Husbandry Activity and relative parotoid gland size. This result is very interesting given that males usually have higher proportions of dorsal yellow than females (Balogová and Uhrin 2015; Preißler et al. 2019; Mühlenhaupt et al. 2025). Models of fire salamander with higher proportions of yellow received fewer bite marks and therefore, more yellow coloration might decrease the risk of predation for a fire salamander (Caspers et al. 2020). Our results could thus indicate that while males might rely on their deterring coloration, females might adjust their activity levels based on the size of their secondary defences, the toxins produced in their skin glands such as the parotoid glands. Similar results have been reported for natterjack toads (*Epidalea calamita*) where males are faster, exhibit more contrasting coloration, have larger parotoid glands, and show less risky behaviors than females due to a greater predation risk for males (Zamora-Camacho and Comas 2017, 2019; Zamora-Camacho 2018, 2022; Zamora-Camacho 2022). In fire salamander males, the dorsal proportion

of yellow might be both selected for by predation and mate attraction (Mühlenhaupt et al. 2025), and therefore, is on average higher in males compared to females, providing an effective defence against most predators (Caspers et al. 2020). In females, the dorsal proportion of yellow might not be selected for by mate attraction, and therefore, is lower in females. This "weaker" defensive mechanism might be compensated in females by either developing larger parotoid glands or reducing activity levels to avoid predator encounters. Future studies should further investigate this sex-difference in the association of chemical and behavioural defences that are driven by diverging selective agents.

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