

Article

Do Active and Passive Antipredator Defences in the Toad *Epidalea calamita* Differ between Males and Females from Natural Habitats and Agrosystems?

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Abstract: Due to their assumed costs, simultaneous antipredator strategies are expected to face trade-offs, which, however, could be milder in individuals subjected to a more intense predator pressure. In this work, I studied the relationship between locomotion and parotoid glands in the natterjack toad, *Epidalea calamita*. Specifically, I predicted that individuals with reduced sprint speed would rely more on their chemical defences, having larger and more aposematically coloured parotoid glands. In addition, I expected this trade-off to be more evident in females and toads from pine grove habitats, because, according to previous work, males and toads from agrosystems are under greater predator pressure. However, sprint speed showed no relationship with coloration, but toads with proportionally greater parotoid glands were also proportionally faster. Thus, the costs of these antipredator traits might not be high enough to make them interfere, or the benefits of simultaneous optimisation of sprint speed and parotoid gland size might outweigh the costs of it in some individuals. In any case, habitat and sex did not affect these relationships, so the trends detected are valid across sexes and the habitats studied.

Keywords: aposematism; *Epidalea calamita*; locomotor performance; parotoid glands; sprint speed



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1. Introduction

Predators are major agents of selection on their prey [1,2]. First and foremost, the future fitness of an individual that is preyed on is reduced to zero [3,4]. Moreover, failed predator attacks may damage fitness in diverse ways [5,6]: they might result in wounds and injuries of varying degrees of seriousness, which may lead to reduced mobility and thus greater susceptibility to future predator attacks [7], or to infections that may compromise health and survival [8]. Last but not least, predator threats can promote the expression of inducible antipredator defences that, be they behavioural [9], physical [10], or chemical [11], usually come with costs, primarily in terms of time [12] or energy consumption [13].

Other antipredator defences are also not devoid of costs. Perhaps along with concealment, the most widespread antipredator strategy in the animal kingdom is flight: when faced with an imminent enemy, most animals actively flee towards the safety of a refuge [14,15]. Accordingly, the available evidence supports a positive relationship between locomotor performance and fitness in multiple animal taxa, in which not only predator avoidance, but also prey capture, dispersal, or intraspecific interactions might be involved [16–18]. However, the muscular exertion linked to locomotion also implies some costs. Energy consumption is probably the most obvious of these [19,20]. Besides, the production of reactive oxygen species as a consequence of the physiological processes involved may unbalance the oxidative metabolism, resulting in oxidative stress [21,22], which may ultimately have noxious effects on physiological homeostasis and animal health [23]. Moreover, particularly in cryptic species, individuals that are in motion have greater chances to

be detected by predators, and thus attacked, than those that remain immobile, so they tend not to flee until detected [24,25].

Other kinds of antipredator defences are no exception to this interplay between benefits and costs. Such is the case of chemical defences, which are poisonous or unpalatable substances that act as deterrents against predators [26–28]. Their benefits notwithstanding, these substances are costly to synthesize, as the metabolic routes involved require both energy and metabolites that may be limiting [29,30]. These chemical defences are oftentimes accompanied by aposematic colorations, which resort to salient colours and patterns that warn potential predators [11,31,32] and discourage them from attacking and/or remind them of past unpleasant experiences, thus avoiding the damage associated with such an attack [33,34]. However, the synthesis of the pigments needed for salient colorations, especially carotenoids and melanin, also incurs well-documented metabolic costs [35,36].

Although simultaneously bearing several antipredator defences could a priori appear as mostly beneficial, their additive costs could be difficult to satisfy. Therefore, animals with various antipredator defences could trade them off. Nonetheless, these trade-offs could be milder when the benefits of these defences outweigh their costs, which could happen under intense predation pressure.

In this work, I tested these predictions on the natterjack toad, *Epidalea calamita* (Laurenti, 1768). This is a cursorial toad that escapes predators by means of quick runs [37]. Moreover, it has prominent parotoid glands. These are a pair of swollen dorsal structures that lay behind the heads of numerous amphibian species, and whose size is directly proportional to the amounts of chemical defences they release [38,39]. The production of chemical defences in the parotoid glands is energetically costly, and trades off with several traits, such as growth rate and dispersal, in other closely related species [40]. In the case of *E. calamita*, these glands are aposematic: the chances of predator attacks decrease with the increasing degree of divergence between parotoid gland and dorsum colour in plasticine models [41]. Moreover, plasticine models exposed in agrosystems receive more predator attacks than those placed in pine grove, supporting the idea that agrosystem toads are under greater predation pressure [41]. Therefore, I predict that both parotoid gland size and colour saliency will be negatively correlated with sprint speed due to energy trade-offs among these. In other words, I expect slower individuals to be more reliant on their chemical defences, thus having greater and/or more salient parotoid glands. However, because predation pressure is greater in agrosystems, I expect these trade-offs to be stronger or only apparent in pine grove toads in comparison, as the former could benefit from greater investment in antipredator defences. Additionally, males in this species have larger parotoid glands than females [41], and are faster [37], which could be responses to greater predation pressure. Indeed, males appear under greater predation pressure in the closely related toad *Bufo bufo* [42]. Therefore, greater exposure to predators may reduce this trade-off in males as compared to females.

2. Materials and Methods

2.1. Study Species

The natterjack toad (*E. calamita*) is a medium-size bufonid (48–82 mm snout-vent length, hereafter, SVL, in the sample studied here) that occurs in vast regions of central and western Europe [43]. Within its distribution area, this species occupies a wide variety of habitats, including natural as well as human-altered landscapes [43]. Given the notable divergence in the climatic conditions among these regions, phenology is subjected to spatial variation: toads hibernate in cold zones but aestivate instead in dry areas [43]. Activity takes place in rainy or very humid, not too cold nights, and the reproductive period may span from winter in warm regions to spring in colder environments [43]. Other than that, toads remain inactive under logs or rocks, or in burrows they dig if the ground is loose enough, where they are sheltered against predators [43]. Natterjack toads are common prey to visual and olfactory predators: snakes such as *Natrix maura* and *N. astreptophora*, birds such as *Larus ridibundus* or *Pica pica* and mammals such as *Meles meles*, among others, are

known to feed on these toads [43]. Commonly, when confronting a predator, toads quickly flee by means of intermittent runs [37]. In other cases, especially if cornered, they bend their bodies and expose their conspicuous parotoid glands, capable of releasing toxins in remarkable amounts [44].

2.2. Toad Capture and Management

Fieldwork took place in Pinares de Cartaya pine grove (SW Spain: 37°20' N, 7°09' W) and surrounding agrosystems. The former consists of an 11,000 ha *Pinus pinea* grove whose understory is dominated by *Pistacea lentiscus*, *Cistus ladanifer* and *Rosmarinus officinalis*. Although the native or introduced origin of this vegetal formation is debatable, it has been predominant in this area for at least the last 4000 years, being thus considered a natural habitat [45]. This pine grove is surrounded by a 2800 ha crop area, some 5 km away, which, during the past decades, has transitioned from extensive vegetable farms to intensive strawberry, blueberry and orange tree (among others) agrosystems. In these agrosystems, landowners add agrochemicals such as fungicides, herbicides, pesticides or fertilizers at their discretion, and summer drought, which spans approximately from May/June to September/October, is tempered by artificial irrigation.

In this area, *E. calamita* reproduces during the winter. Therefore, toad capture took place from December 2018 to March 2019. In total, I captured 25 males and 21 females in the pine grove, plus 20 males and 22 females in the agrosystem. I caught these toads by hand while they were active on rainy nights, after which I transported them to the laboratory in plastic buckets with a lid with holes and damp earth as a substrate. Once in the laboratory, I sexed these toads based on colour sexual dimorphism (males are greener and have pinkish or purplish vocal sacs, whereas females are browner and have greyish throats [46]) and on the presence of dark and rough nuptial pads only in males [43]. Then, I housed the toads in individual plastic containers (20 × 13 × 9 cm) with humid peat substrate and a piece of opaque plastic as a refuge.

2.3. Coloration and Morphological Measurements

Within 24 h from capture, I measured their SVL with a ruler, to the nearest mm, and I took an orthogonal photograph of each toad's dorsum with a photo camera Canon EOS 550D at a maximum resolution of 18 megapixels, with a shutter-aperture of F10 and a fixed focal length of 53 mm. Exposure time, by contrast, was automatically adjusted by the device to optimise colour capture in each case. Perpendicularity and stability were assured with a tripod which allowed a steady distance of 40 cm between the lens and the photographed surface. This surface consisted of a horizontal square (30 cm side) of white paper. Three vertical white polyester squares (30 cm side) sat respectively on both sides and the rear of this surface, turning it into an incomplete cube where the front and the upper sides were missing: the open front side allowed toad handling, and the open upper side allowed photographs from above. Externally from each lateral polyester square, an 80 W white-light bulb at a height of 20 cm provided the scene with bidirectional illumination that prevented shades. Plus, the fact that those white-light bulbs filtered by white polyester were the only light sources in the room (photos were taken at night, in a completely closed room) eliminated any parasitic light that could have altered colour recording. After fixing the camera to the aforementioned parameters, but before photos were taken, white balance was calibrated to a clean piece of paper. Then, a piece of graph paper for biometric measures and a standardised colour chart (IMAGE Photographic) for colour calibration were placed on the photographed surface, close to the toad itself. Dust and humidity were removed from toads' skins with a disposable napkin prior to each photo.

These photos were then analysed with the software Adobe Photoshop CS5, after calibrating white balance again in each photo by applying the grey eyedropper in the white balance calibration function on the colour chart. Moreover, I set the colour mode to the L*a*b* three-dimensional colour space described by the Commission Internationale d'Eclairage (CIE) [47]. In this colour space, L* is a measure of lightness, and spans from

0 (pure black) to 100 (pure white), a^* represents the green–red axis (negative values are green and positive values are red) and b^* represents the blue–yellow axis (negative values are blue and positive values are yellow). Afterwards, I used the piece of graph paper to calibrate length, and manually delimited both parotoid glands with the lasso tool so as to calculate the sum of both parotoid glands' area. Next, I averaged the colours of each parotoid gland as previously selected and used the histogram tool to gather the average L^* , a^* and b^* values of both. Finally, following the same methods, I demarcated the dorsum, leaving the parotoid glands out, averaged its colour and gathered its L^* , a^* and b^* values. With these values, I calculated parotoid gland colour saliency (ΔE^*) by applying the formula $\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{1/2}$ to L^* , a^* and b^* parameters of parotoid glands and dorsum, as the CIE proposes [48,49].

2.4. Sprint Speed Measurement

Sprint speed trials were conducted 48 h after these photos were taken. Toads spent this time in the individual plastic containers previously described. One hour before the trials, I gently but firmly pressed each toad's lower abdomen to empty their bladders [50,51]. In this way, I reduced to zero, and thus standardised, bladder water burden, which could affect locomotion [50–52]. Then, toads were allowed to rest in their containers for one hour, after which I recorded them (with a video camera Canon EOS 550D, set at 25 frames per second) while individually running in a brown cardboard linear runway ($200 \times 15 \times 15$ cm). The bottom of this runway was divided into 10 cm stretches with perpendicular white stripes whose colour contrasted with the brown cardboard, so that both limits of each stretch could be accurately discerned in the video. The cardboard surface was rough enough to favour traction, which is relevant because the substrate may affect locomotion [53]. Moreover, a black background at one end of the runway was intended to be mistaken for a shelter and encourage toads to move forward. Toads were released at the other end of the runway, and manually chased constantly but gently, in order to stimulate their moving forward, until they reached the end of the runway. Since temperature may affect amphibian motility [54], room temperature was roughly constant (19°C) throughout the process. Trials were always conducted at night, when toads are naturally active. Illumination was also standardised with a 60 W bulb located 2.5 m above the centre of the runway. After the trials, I released the toads at their capture sites as soon as possible. Toads suffered no visible damage as a consequence of this study.

Then, I used the software Tracker v. 4.92 for frame-by-frame video analysis. Specifically, I recorded the time (precision: 0.01 s) toads used to cover every stretch, i.e., the time toads spent between the perpendicular strips delimiting both ends of each stretch [37,55,56]. Since stretch length was known (10 cm), I could calculate the speed (in cm/s) of each toad in each stretch by dividing 10 cm by the time (s) needed to cover it. Finally, I considered the fastest value of each toad as its sprint speed.

2.5. Statistics

In order to control for the effect of body size on parotoid gland area and on sprint speed, I calculated the residuals of the simple regressions of parotoid gland area and of sprint speed on SVL, whereas parotoid colour saliency was analysed untransformed. After checking the assumptions of homoscedasticity and residual normality, I applied parametric statistics [57]. The residuals of sprint speed on SVL needed to be square-root-transformed so as to be homoscedastic. Data were analysed with the software Statistica v8.0.

Firstly, I conducted an ANCOVA, where the residuals of sprint speed on SVL (square-root-transformed) were the response variable, the residuals of parotoid gland area on SVL were the covariates and habitat and sex were included as factors. The interactions among all covariates and factors were included in the full model (presented as Supplementary Material), to which stepwise backward selection was applied.

Then, I performed a similar ANCOVA where the residuals of sprint speed on SVL (square-root-transformed) were the response variable, parotoid colour saliency was the

covariate and habitat and sex were included as factors. The interactions among all covariates and factors were included in the full model (presented as Supplementary Material), to which stepwise backward selection was applied.

3. Results

3.1. Parotoid Gland Area

The full model detected that the residuals of sprint speed on SVL were greater in males than in females, and positively related to the residuals of parotoid gland area on SVL, whereas the effects of habitat and all the interactions were non-significant (Tables S1 and S2, Supplementary Material). The final model after stepwise backward selection maintained the effects of sex ($F_{1,85} = 9.508$; $p = 0.003$) and the residuals of parotoid gland area on SVL ($F_{1,85} = 10.090$; $\beta = 0.310$; $p = 0.002$; Figure 1).

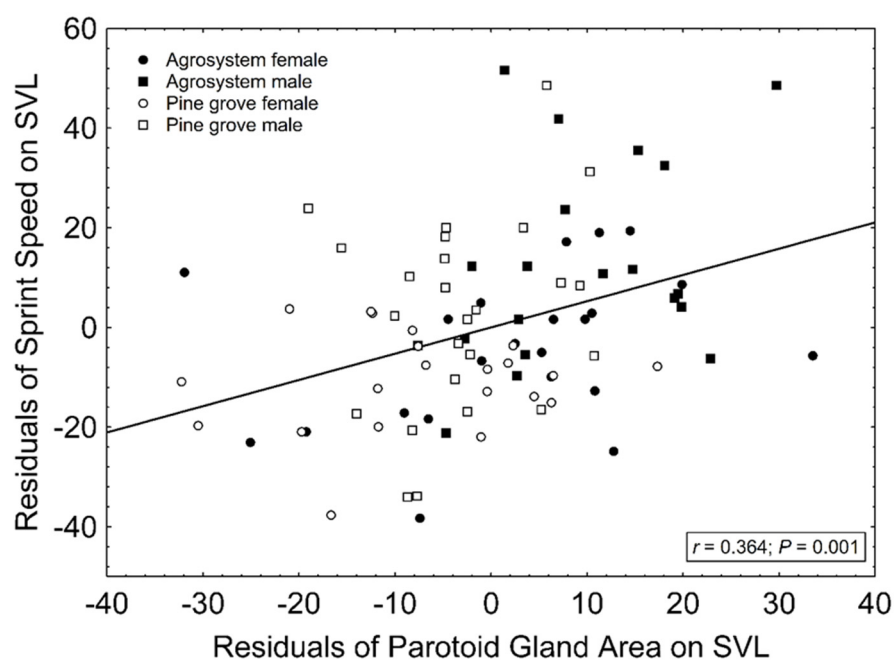


Figure 1. Correlation between the residuals of sprint speed on SVL and the residuals of parotoid gland area on SVL. r and p values are indicated.

3.2. Parotoid Gland Colour Saliency

The full model detected no effect of either of the factors, the covariate, or their interactions on the residuals of sprint speed on SVL (Tables S3 and S4, Supplementary Material). In the final model after stepwise backward selection, only the effects of sex ($F_{1,85} = 14.214$; $p < 0.001$) and habitat ($F_{1,85} = 4.049$; $p = 0.047$) were significant.

4. Discussion

These results challenge both the fundamental predictions on which this work was based. To begin with, they provide no evidence of a trade-off between sprint speed and parotoid gland colour saliency or size. Contrary to what was expected, slower toads do not invest more in colour signalling of their parotoid glands. What is more, whereas sprint speed appears unrelated to parotoid gland colour saliency, it is directly proportional to parotoid gland size. Not only does such a result not match the expectations, but it reverses them: individuals that are faster also have larger parotoid glands. Therefore, some toads are allegedly better suited against their predators and simultaneously optimise both these characteristics.

The absence of a trade-off between these antipredator strategies could imply that their costs are not high enough to interfere with each other's development. Remarkably, locomotion also remains unaltered despite immune system activation in other amphibians, which

might highlight its antipredator worthiness [58]. Indeed, evidence has been detected that prey locomotion mode affects other antipredator strategies, such as the escape tactic [59]. An alternative, but non-mutually exclusive explanation, could be that individuals under greater predation risk offset or even overcompensate the potential trade-offs among these traits by increasing food consumption, as has been observed in birds [60]. Indeed, food restriction exacerbates energy-based trade-offs in nematodes [61].

Additionally, the fitness benefits of maximizing sprint speed (assuming that its antipredator role does not conflict with its other roles, for example, in food acquisition, dispersal, or intraspecific relationships) and parotoid gland size at the same time could outweigh the energy costs of it in individuals under greater predation risk. Previous pieces of research have independently established that, in this system, *E. calamita* males are faster [37] and possess larger parotoid glands [41] than females. Moreover, these glands are larger in agrosystem than in pine grove individuals [41]. Extensive scientific literature on this [37,41,62,63] and other species [64,65] suggests that male toads are more conspicuous than females, probably as a consequence of male competition for females, and thus might undergo greater predation pressure [42]. Moreover, plasticine models of toads receive more predator attacks in agrosystems than in pine grove in this system [41]. Therefore, it could be assumed that the positive correlation between sprint speed and parotoid gland size could be a consequence of males being faster and possessing larger parotoid glands than females. However, this effect was already present in the full ANOVA prior to stepwise backward selection, where sex, habitat and their interactions with the residuals of parotoid gland size were controlled for and proved non-significant. Therefore, the positive relationship between sprint speed and parotoid gland size is valid across sexes and habitats rather than a mere by-product of sexual dimorphism in speed and parotoid gland size and/or habitat divergence in the latter. This result contrasts with findings on other taxa, such as *Centruroides vittatus* scorpions, where slower individuals make more frequent use of their venomous stings, which leads to sexual dimorphism in antipredator responses: females, which are slower, tend to attack with their stings at greater rates than males, which are faster and tend to flee more often than females [66].

Moreover, unlike the case herein, antipredator strategies and the relationships among them are known to be subjected to spatial variation in numerous species, according to predator pressure (reviewed in [67]). Specific antipredator strategies [68] and the relationships among them can vary according to the actual predation risk [69]. However, constraints to such spatial adjustments may exist, either genetic- or trade-off-based [67]. Although the reasons remain obscure, in the case studied here, however, there is no evidence of spatial variation in the relationships (or the lack thereof) of sprint speed with parotoid gland size and coloration.

To conclude, no trade-off could be detected between sprint speed and parotoid gland size or coloration. Instead, sprint speed showed no relationship with coloration, but a positive one with the size of these glands, which reverses the expected trend. This finding could imply that the costs of these antipredator strategies are not high enough to make them interfere, or even that the benefits of the simultaneous optimisation of sprint speed and parotoid gland size outweigh the costs of it in some individuals. In any case, habitat and sex do not affect these relationships, which suggests that the trends detected are valid across sexes and the habitats studied, with no evidence of separate evolution.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/d13120614/s1>: Tables S1–S4; Dataset.

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