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Alternative strategies and physiological trade-offs in a polymorphic lizard

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Summary

INTRODUCTION

COLOR POLYMORPHISM

One of most fundamental goal in evolutionary biology is the understanding of the process that generate and maintain genetic polymorphism (Levene, 1953; Crow & Kimura, 1972). The heritable visible markers phenotypes, i.e. color polymorphism, provide for the ecologist and the evolutionary biologist opportunities to investigate and understand fundamental evolutionary processes such as natural and sexual selection, genetic drift and gene flow (Ford, 1945; Chunco, McKinnon & Servedio, 2007; Svensson et al., 2009; Svensson, McAdam & Sinervo, 2009). Although the development of enzyme electrophoresis and molecular biology made it possible to score vast amount of genetic variation at many loci in natural populations (Lewontin, 1977; Svensson, 2017), heritable color polymorphism (CP) constitutes ideal model systems to study evolutionary processes for several reasons. CP has a simple genetic basis and shows high heritability and more simply it can be scored unambiguously in a large number of individuals and thus provide easy visual markers for examining selection in the wild (Wellenreuther, Svensson & Hansson, 2014). The presence of different genetically-based polymorphisms within populations may promote speciation, so CP provides us clues to speciation processes, either in allopatry or (more controversially) in sympatry (West-Eberhard, 1983; Jonsson & Jonsson, 2001; Gray & Mckinnon, 2006; Corl et al., 2010; Hugall & Stuart-Fox, 2012; McLean & Stuart-Fox, 2014). Studies over many vertebrate species suggest the evolution of multiple variable morphs as an impetus for speciation (Allender et al., 2003; Seehausen & Schluter, 2004; Corl et al., 2010; Hugall & Stuart-Fox, 2012).

CP occurs when in a population, individuals of the same age and same-sex display one or more color morphs genetically determined whose expression is neither sensitive to the environment nor to body condition, and the rarest morph being too common to be the product of recurrent mutations (Huxley, 1955; Buckley, 1987). It is a phenomenon with extensive documentation in both invertebrates and vertebrates, across the tree of life (Mckinnon & Pierotti, 2010; McLean & Stuart-Fox, 2014; Wellenreuther, Svensson & Hansson, 2014). The presence of different morph, genetically determined, within a population may promote speciation (McLean & Stuart-Fox, 2014): phenotypic variation among morphs gradually accumulates, and over time isolated populations with different morphs will diverge, facilitating speciation (West-Eberhard, 1986). Within a given population, CP is generally associated with the coexistence of alternative reproductive strategies, which are modulated by complex interactions among environmental pressures (e.g. social interactions and individual density), each one involving specific trade-offs among behaviour, physiological, and other life-history traits (Roulin & Bize, 2007). For example, in cichlid fish, male aggression is primarily directed towards males of the most common colour, and rare colour morphs enjoy a negatively frequency-dependent fitness advantage. (Seehausen & Schluter, 2004). The CP increases speciation rates in birds (Hugall & Stuart-Fox, 2012) and reptiles (Corl et al., 2010). Therefore, alternative strategies among morphs may lead to evolution of morph-specific optimal trait combinations, which represent alternative solutions to fitness optimization in the face of trade-offs in resource allocation (Sinervo 2000; Roulin 2004; Sinervo & Lively, 1996; Svensson, Sinervo & Comendant, 2001; Roulin & Bize, 2007).

TRADE-OFF

Trade-offs are usually thought in terms of resource allocation, where the allocation of limited resources to one trait has negative consequences for the other traits requiring the same resource (Zera & Harshman, 2001). The ‘Y-model’, which stated that for a given amount of resources it is impossible to increase two or more life-histories traits at once, is one way to describe how mechanism of trade-offs works (Zera & Harshman, 2001; Garland, 2014; Martin, 2015). In this framework, every time a characteristic enhances one aspect of performance it will decrease anoter type of performance (Garland, 2014). Correlations between size and number of eggs that birds, fishes or reptiles lay in a clutch are a typical example of different strategies within the same population (Elgar, 1990; Sinervo & Licht, 1991; Martin et al., 2006). In the past decades, ecologists and evolutionary biologists have focused their interest on physiological trade-offs: particularly the correlation between immune function and hormones. The immune system is the animal's defence mechanism to fight or control any parasitic or pathogenic infection (Lochmiller & Deerenberg, 2000), and hormones, such as testosterone, modulate aggressive behaviour , territorialism (a risk-taking behaviour), and in general affect all those characteristics that promote and favour reproductive success (Oliveira, 2004; Hau, 2007; Huyghe et al., 2009; Negro et al., 2010; Mokkonen et al., 2012). One of the most important trade-offs that includes immune function and hormones was one stated by ImmunoCompetence Handicap Hypothesis (ICHH, Folstad & Karter, 1992), which explains that testosterone is a 'double-edged sword', developing characteristics used in a reproductive context but at the same time reducing the response of the immune system and thus individuals survival (Marler & Moore, 1988). High testosterone plasma level can decrease immune functions, favours parasite infections, stimulates risky behaviours, and thus diminishes survival (Olsson et al., 2000; Klukowski & Nelson, 2001; Cox & John-Adler, 2007). Therefore, no way for males of maximizing stamina and aggressive behaviours at once. According to the ICHH, male sexual ornaments provide honest signals of the males' immune function due to the trade-off between ornament development and immune health via the effects of testosterone. Ornament development triggers a down-regulation of immune function, which is effected through the immunosuppressive effect of testosterone. This immunosuppression makes it impossible for males of low genetic quality to develop exaggerated ornaments without compromising their health and potential future reproductive success. Thus, only high-genetic-quality males can afford to sustain the high levels of testosterone required for the development of elaborate ornaments (Foo et al., 2016). ICHH was demonstrated in several studies over different vertebrate taxa (Roberts, Buchanan & Evans, 2004; Mills et al., 2008; Foo et al., 2016).

GOOD MODEL: REPTILES

Understand physiological trade-offs in wild animal needs for model species that allows testing alternative solutions to trade-offs in lab under strictly controlled experimentally conditions.

Reptiles, and in particular lizards, are particularly useful models for this kind of research (Smith & French, 2017), due to the own biology proprieties, such as:

i) body temperature; lizards are ectotherm, so their body temperature is easy enough to maintain in optimal levels for immune tests (Sacchi et al., 2014. Sannolo et al., 2014), and the behavioral thermoregulation of free-living individuals can be correlated with other measures of performance (Taylor, DeNardo & Malawy, 2004; Smith & French, 2017);

ii) discrete reproductive investment; most of lizard species lay eggs (~85%, Tinkle, 1967) and we can measure the reproductive investment by analyzing the composition of the yolk and other elements (Congdon & Gibbons, 1985; Sacchi et al., 2012; Smith & French, 2017);

iii) predictable seasonal patterns; temperature and water availability lead animals to have a period for reproductive activity, and periods to restore or preserve energy reserves (Moritz et al., 2012). This allows us to predict, test and measure the effects of reproductive investment on immunity (Smith & French, 2017);

iv) territoriality and ease of housing; it is quite easy to catch animals and their site fidelity allows us to study individuals for many years (with capture/recapture methods, Sacchi et al., 2010). Furthermore, many lizards are small and can be easily housed in terraria for lab studies, in addition herpetocultural is common and developed (Smith & French, 2017).

Since the last decades, studies on the maintenance of color polymorphism have been increasingly using lizards as model species (Gray & Mckinnon, 2006). For example, *Uta stansburiana*, the side-blotched lizard, is one of the best case-study of color polymorphism. Males of these lizards show three colors on throat and belly, blue orange and yellow, which are associated with as many alternative breeding behaviours. In fact, they play a “rock-paper-scissor” strategy, maintained by the interaction between frequency-dependent selection and among-year changes of morph fitness (Sinervo & Lively, 1996). Orange males (OMs) are polygynous, more aggressive and defend large territories, which overlap with several females’ territories, and they are considered ‘ultra-dominating’. Blue males (BMs) are less aggressive and defend small territories, and they are considered ‘mate-guarders’. Finally, yellow males (YMs) do not defend territories, mimicking females' morphs and they are considered ‘sneakers’. OMs’ strategy is defeated by the sneakers YMs’ strategy and defeated by the mate guardian BMs’ strategy, while YMs strategy is in turn defeated by mate guarder BMs’ strategy (Sinervo & Lively, 1996). Moreover, in males of the tree lizards, *Urosaurus ornatus*, it has been demonstrated that the colormorph predicts encounter outcome more than size: even though yellow males are bigger, green males are more likely to dominate (Hover, 1985). The same occurs in males of Australian painted dragon, *Ctenophorus pictus*, where red morphs win more likely contests against yellow ones (Healey, Uller & Olsson, 2007).

COMMON WALL LIZARDS (*Podarcis muralis*)

For my thesis, I choose as model species the Common wall lizard (*Podarcis muralis*), a medium-sized diurnal lizard of central and south-eastern Europe (50–70 mm adult snout-vent length, SVL) (Sacchi et al., 2007b; Sillero et al., 2014), which shows three main colour morphs in both sexes, white, yellow, and red, on throat and belly (Sacchi et al., 2007b). In the last decades, many researches focused on the role of CP in social communication, its evolution and maintenance on this lizard. The morphs are genetically controlled (Andrade et al., 2019), and ontogenetically stable once they develop from the second year of life (Cheylan, 1988; Pérez i de Lanuza, Font & Carazo, 2013). Frequencies of morphs in Northern Italy are highly variable among population (Sacchi et al. 2007). Females of this species play r-K strategies, where yellow morphs are r-strategists, white morphs are K-strategists and red morphs follow a mixed strategy (Galeotti et al., 2013). In general, each morph is characterized by different phenotypic traits, i.e. body size, immunity, homing behaviour, diet, stress response, chemical communication and plasma T levels (Sacchi et al., 2017b, 2007b,a; 2017a; Martín, Amo & López, 2008; Calsbeek, Hasselquist & Clobert, 2010; Galeotti et al., 2010; Scali et al., 2013, 2016; Pellitteri-Rosa et al., 2014; Mangiacotti et al., 2017, 2019b,a). Correlation between morphs and aggressiveness remains controversial:

according to Sacchi et al. (2009), simple rules, such as residency and body size difference, determined outcome of agonistic encounters, but no effect of morphs was found in aggressive response or fighting success. Experimental test in neutral arena, however, found that the extent of black patches on the belly emerged as a good predictor of contest outcome independently of morphs, even if red males lost fights against heteromorphic males more often than yellow or white males (Abalos et al., 2016). This effect, nonetheless, could be due to a correlation with the size of black patches.

Very interesting is the presence of a morph specific pattern of immune response and plasma T levels throughout the breeding season. Yellow males have higher plasma T levels at the early breeding season (April), and lower at the end (July) (Sacchi et al., 2017b) but they are also more immunosuppressed (Sacchi et al., 2007a; Galeotti et al., 2010) compared to the other morphs.

THESIS AIMS AND OUTLINE

All this scientific background allowed me to hypothesize a physiological trade-off between immunity and hormones (therefore also to aggressiveness). Yellow males could play a different strategy compared to the other colour morphs. That is yellow males invest more energy in aggressive interactions and intrasexual-competition at the beginning of the breeding season, at the expense of better stamina (as stated by ICHH); consequently, they are expected not to be able to maintain the needed aggressive level also in the late season (Sacchi et al., 2017b).

To assess my hypothesis I have to test both aggressive- and immune-response of males. For the latter test I used research available in the literature on lacertids, and particularly in *P. muralis* (Oppliger et al., 2004; Sacchi et al., 2007a, 2014). For the former test I had to overcome some problems first. In fact, I am not interested in analysing aggressiveness at individual level, which can be actually modulated by the specific experience of each individual, in simple terms is affected by individual personality. I would focus on aggressiveness at morph levels: in that case, all individuals of same morph share the same aggressive response, due to the same strategy played which is genetically determined as well as colormorph.

The first part of my thesis (**Chap. 1, 2, 3**) represents the method with which I solved this issue. In **Chap. 1** I demonstrate the role of visual stimuli in territorial communication, but only as a combined effect of shape and motion, using mirrors that mimicks a stranger intrusion in the individual's territory. Mirrors allow removing the size and motivation effects by presenting to the focal individual an image keeping the same behaviour, size, and motivation (Sacchi et al., 2009).

With **Chap. 2**, another issue was verified: since the mirror reflects an image of the same morph of focal individuals, I wanted to be sure how morphs could affect aggressiveness in males comparing the aggressive response among morphs. Thus, I manipulated the ventral color of males and I assessed if aggression varies among morphs under two contrasting hypotheses: a heteromorphic vs. a homomorphic aggression. After lab test, I tested these two hypotheses again in a wild population with a field experiment, measuring the mean distance between morphs and analysed spatial distribution. If males have a heteromorphic aggressiveness, they will occupy the space with similar patters; on the contrary, if aggressiveness is most directed against males belonging to the same morphs, lizards will tend to minimize the risks due to the conflict, so males will be distributed following the 'dear enemy' phenomenon (neighboring males have different morphs) (Whiting, 1999).

The last question that I had to consider was how the aggressive response measured by the mirror depended on the housing condition (**Chap. 3**). Thus, I investigated how aggressive behavior varies according to a change in subjective Resource Value (RV) and color morphs, while controlling for the asymmetry in Resource-Holding Potential (RHP). To do this I compared aggressive response of the same individual towards its mirror image in two different arenas (familiar = high subjective RV; novel = low subjective RV).

After validation of the aggression measurement method, by **Chap. 4** and **5** I tested my prior hypothesis, either correlatively and experimentally respectively.

In **Chap. 4** I measured aggressive response to conspecifics of yellow and white morphs throughout the breeding season (from April to July, 117 trials), in order to assess if males modulated aggressive response by two strategies: a 'risky strategy' yellow males and 'conservative strategy' white males. I considered three types of aggressive response, with different levels of aggressiveness: (i) bite against the image reflected in the mirror, (ii) time spent by the individuals into the half mirrored cage, and (iii) number of times the lizard entered the half mirrored cage. We also considered the number of tongue flicking as explorative behaviour variable.

Therefore, in **Chap. 5** I used an experimental approach to test the presence of the two strategies, hypothesized in the Chap. 4, manipulated the plasma T levels of males in order to challenge the immunosystem and analysed the effect on males. Hence, I measured aggressiveness and immune response before and after hormonal treatment and verified if the two morphs are two opposite settings of the ICHH.

All chapters (1-5) are manuscripts already published or submitted, waiting for the last reviews. Therefore, some repetitions cannot be avoided, and the overall structure may follow a too self- supporting rationale. I am the primary author for **Chap. 4** and **5**, and in **Chap. 1-3** I am one of the most contributors. All co-authors are informed and gave their permission to include the manuscript in the thesis. **Chapter 1, 2, 3**and **4** are published in journal articles, they are included according to the journal policy (CC-BY 4.0). These are:

- Scali S, Sacchi R, Falaschi M, **Coladonato AJ**, Pozzi S, Zuffi MAL, Mangiacotti M. 2019. Mirrored images but not silicone models trigger aggressive responses in male Common wall lizards. Acta Herpetologica 14:35–41. DOI: 10.13128/Acta.
- **Coladonato AJ**, Mangiacotti M, Scali S, Zuf MAL, Pasquariello C, Matellini C, Buratti S, Battaiola M, Sacchi R. 2020. Morph-specific seasonal variation of aggressive behaviour in a polymorphic lizard species. PeerJ:1–16. DOI: 10.7717/peerj.10268.
- Scali S, Mangiacotti M, Sacchi R, **Coladonato AJ**, Falaschi M, Saviano L, Rampoldi MG, Crozi M, Perotti C, Zucca F, Gozzo E, Alberto M, Zuffi MAL. 2020. Close encounters of

the three morphs: does color affect aggression in a polymorphic lizard? DOI: 10.1002/ab.21961

- Sacchi R, **Coladonato AJ**, Battaiola M, Pasquariello C, Mangiacotti M, Matellini C, Buratti S, Scali S, Zuf MAL (under review) Subjective resource value affects aggressive behavior independently of Resource-Holding-Potential and color morphs in male common wall lizard. *JEthol* (2021). https://doi.org/10.1007/s10164-021-00690-6

Chap. 5 is a ready to submit manuscript:

- **Coladonato AJ**, Mangiacotti M, Scali S, Zuf MAL, Salgarella G, Pasquariello C, Matellini C, Buratti S, Battaiola M, Sacchi R. (*in prep*). Effect of testosterone manipulation on immunocompetence and aggressiveness in males polymorphic lizard.

CHAPTER 1

Mirrored images but not silicone models trigger aggressive responses in male Common wall lizards

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Abstract. Disentangling the effects of single releasers in animal communication is a demanding task because a releaser often consists of a combination of different key stimuli. Territorial communication in reptiles usually depends on visual, chemical, and acoustic stimuli, but the role of each of them depends on phylogeny. Lacertids are modern lizards that rely mainly on chemical cues for their communication, but they also use aggressive displays based on visual recognition. We experimentally tested the visual stimuli that release an aggressive response in the males of a typical lacertid, the common wall lizard (*Podarcis muralis*), testing the effects of silicone models and mirrored images in captivity. The response to models and control (a blank sheet) was not significantly different and these stimuli did not release any aggressive behaviour. On the contrary, the reflected image in a mirror caused overt aggression (i.e., bites against it) in 63% of tested individuals. The results clearly demonstrate the role of visual stimuli in territorial communication, but only as a combined effect of shape and motion, differently from other lizard families for which shape is enough to stimulate aggressive responses. Mirrors can be useful tools to investigate aggression related to physiological and morphological aspects in lacertid lizards.

Keywords. visual stimulus, aggression releaser, mirrored image, plaster model, Lacertidae.

INTRODUCTION

Animal communication systems have evolved so that individuals can make decisions based upon the behaviour, morphology, and physiology of other individuals (Endler, 1993). Communication depends on signal transmission between a signaller and a receiver and in order for the signal to be successful, it must be detected by the receiver against a background of other potential stimuli (Fuller and Endler, 2018). Signals act as releasing mechanisms, intended as the totality of all parts of the nervous system that are involved in the filtering of incoming stimuli and it ensures that only the "appropriate" stimuli release a specific behaviour pattern (Immelmann, 1983). The key stimulus or releaser may consist of single or complex cues and disentangling their effect to fully understand which of them releases certain behaviours could be challenging. The majority of releasers consists of a combination of motor patterns acting as a signal and a morphological structure enhancing the signal's effect (Lorenz, 1981), so a single portion of a key stimulus could be insufficient to release a particular behaviour. Notably, when the behaviour pattern is simple and the risk of error is low, releasers are extremely simplified, such as the pecking at the red spot on the beak triggering the regurgitation of food by adult herring gulls (Tinber-

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gen, 1951). By contrast, when costs associated with errors are high, the releasers are more complex to ensure that behavioural patterns start only when they are necessary. This especially applies to aggressive behaviours, whose primary function is releasing of aggression against fellow members of the species and to avoid unnecessary fighting against heterospecific opponents (Immelmann, 1983).

Aggressive behaviours are most common in territorial species, because individuals are forced to compete for limited resources, such as partners, food, shelters and reproductive sites, even if territoriality can be also assessed without overt aggression (Brown, 1964; Myrberg and Thresher, 1974; Van den Berghe, 1974; Stamps, 1977; Kaufmann, 1983). Among reptiles, many cases of territorialism are well documented for chelonians, crocodiles, tuataras, and, particularly, for lizards (Pough et al., 2004). Three main kinds of stimuli can be used for territorial communication by reptiles: visual, chemical, and acoustic. The latter is typical of species living in habitats where visual displays are ineffective and it is used only by nocturnal geckoes (Marcellini, 1977), terrestrial tortoises (Galeotti et al., 2005a; Galeotti et al., 2005b) and crocodilians (Vliet, 1989). Chemical cues are effective communication tools, used by most reptiles, but particularly developed in modern species, like snakes and most scleroglossan lizards, thanks to the evolution of a complex vomeronasal system that freed the tongue from its ancestral role and allowed the transformation to a chemosensory organ (Mason, 1992; Schwenk, 1993; Cooper, 1994).

Disentangling the role of intraspecific communication channels needs experiments analysing the single cues separately. For example, numerous studies have been done on iguanians (in particular on agamids and iguanids), a reptile clade that bases most of its territorial communication on visual stimuli, like posture, dewlap extension, colour patches and colour changes (Yang et al., 2001; Yang and Wilczynski, 2002; Van Dyk and Evans, 2008; Norfolk et al., 2010; Osborne et al., 2012; Dunham and Wilczynski, 2014; Yewers et al., 2016). On the contrary, information is still lacking on the role of visual cues in lacertids, a family of scleroglossan lizards that relies mostly on chemical communication by means of femoral pores to assess territoriality (Cooper, 1994; Martin and López, 2015; Mangiacotti et al., 2017; Baeckens et al., 2018).

Visual stimuli can be tested using four kinds of methods: silicone models that mimic shape and colours of the species, the reflected image in a mirror of the subjects involved in the experiments, video playbacks showed to lizards, and direct staged encounters with another male. The latter method has been often used, but many factors can affect results, such as opponent size, residence status, and individual motivation (Sacchi et al., 2009). Video playbacks have been successfully used for some species (Macedonia and Stamps, 1994; Yang et al., 2001; Ord et al., 2002; Van Dyk and Evans, 2008; Frohnwieser et al., 2017), but they require a long preparation time to acquire video sequences or to prepare animated images after 3D scanning. Hence, it requires a substantial *a priori* knowledge of the species' stereotyped behaviours to present a complete sample set to the subjects. Silicone models have been used, for example, with *Stellagama stellio* and *Pseudotrapelus sinaitus* (Norfolk et al., 2010), *Platysaurus minor* and *P. monotropis* (Korner et al., 2000) and they proved to be adequate cues for territorial behaviours both for agamids and cordylids.

Mirrors are the most used visual stimuli in experimental designs because they are easy, cheap, and typically stimulate aggressive behaviours against the reflected image (Balzarini et al., 2014). Furthermore, the signal is enhanced by positive feedback, because an aggressive posture or behaviour is immediately replicated by the mirrored lizard. Numerous species have been successfully tested using this methodology, particularly from families Agamidae, Phrynosomatidae, and Dactyloidae (Korzan et al., 2000; Brandt, 2003; Farrell and Wilczynski, 2006; Osborne et al., 2012; Dunham and Wilczynski, 2014).

Podarcis muralis, our model species, is a typical representative of this family. It is a small (snout to vent, SVL up to 7.5 cm) and sexually dimorphic lizard, with males stouter and with bigger heads than females; males show a marked territorial behaviour (Edsman, 1990; Sacchi et al., 2009), as supported also by data about testosterone levels and homing behaviour in previous works (Scali et al., 2013; Sacchi et al., 2017). As a consequence, intraspecific communication in this species has been intensively studied as far as chemical cues are concerned (Martin et al., 2008; Heathcote et al., 2014; Pellitteri-Rosa et al., 2014; Baeckens et al., 2017; Mangiacotti et al., 2017, 2019), but no information is available about visual stimuli and communication during intraspecific encounters (but see Zagar et al., 2015). Since aggressive displays and postures of *Podarcis muralis* have never been detailed before (but see Sacchi et al., 2009 and Abalos et al., 2016 for some information), we discarded playback videos and chose a dual experimental approach based on silicone models and mirrors as visual stimuli to boost and record aggressive behaviours. The specific aim of our work was to assess if visual stimuli can trigger an aggressive response in a typical lacertid lizard that bases most of its intraspecific communication on chemical cues. We did this by comparing the aggressive response to: i) a static and oversimplified visual cue (i.e., a silicone model); ii) a more complex and realistic visual stimulus combining movement, behaviour, and posture (i.e., a mirrored image).

MATERIALS AND METHODS

Ninety *P. muralis* adult males (SVL > 50 mm) were captured by noosing in various localities in Lombardy (northern Italy) between April and June 2016, to maximize territorial response in accordance with reproductive season (Corti and Lo Cascio, 2002; Sacchi et al., 2017). The capture sites were located within 50 km each other and had similar ecological conditions, being all peripheral urban habitats, with comparable habitats, presence of predators and densities. Lizards were carried to the Natural History Museum of Milan and housed in individual plexiglas boxes (40 × 40 × 30 cm) with a refuge positioned near one box's wall, water *ad libitum* and fed with three mealworms (*Tenebrio molitor*) per/day. A sheet of absorbent paper was used as substrate, to keep the resident odour in each terrarium and thus lizards could consider it as their own territory. The vertical sides of the boxes were also covered externally with white paper sheets, to avoid external stimuli and wall reflectance. The room was exposed to a natural day/night cycle.

After an acclimation period lasting between three and seven days, lizards were tested in the same terrarium where they were kept, after removing water and food. A heating lamp (ZooMed Repti Basking spot lamp, 150 W) was turned on for 15 minutes to achieve a plateau body temperature similar for all the individuals (Sannolo et al., 2014), then it was turned off and a cold led lamp (Greenergy, 8 W, 600 lm) was lighted to ensure uniform lighting in the terrarium. A surveillance camera (SONY Super Night Vision Camera, M020-s53-001, located near the heating lamp) was turned on and lizard behaviour was recorded for 15 minutes immediately after inserting a visual stimulus in the box. Individuals were randomly assigned to the following three different stimuli (n = 30 for each treatment without replicates): i) a white paper sheet covering one side of the box, and used as control to simulate the insertion of an object by researcher's hand; ii) a silicone model simulating a new lizard invading the resident's territory; iii) a mirror covering one side of the box, reflecting lizard image and movements. All the stimuli were positioned inside the terrarium near the wall of the box opposite to the refuge.

The lizard model was a silicon-rubber cast prepared by the museum taxidermist using a dead male *P. muralis* specimen that was painted brown on the back and white on the belly and throat using water-based tempera colours (Fig. A1 in Supplementary materials). The model was painted one month before starting the experiment to perfectly dry the paint. A push-up stance was obtained inserting an iron wire in the model to simulate a territorial posture with the anterior part of the body raised and showing throat colouration (Molina Borja, 1981).

Since difference in individual size has been proved to affect the outcome of male-male combats in *P. muralis* (Sacchi et al., 2009) and the lizards tested against the model did not always had the same size as the model (SVL = 67 mm, see Table A1 in Supplementary materials for lizard mean size), we performed a preliminary test to investigate the potential inhibitory effect of such a difference. Latency (i.e., the time between the insertion of the model in the terrarium and the first movement of the focal lizard) was used as a proxy for the potential inhibitory effect (the longer the latency, the larger the effect) and it was regressed against the signed difference between lizard and model size ($SVL_{lizard} - SVL_{model}$). The regression was not significant (one-way ANOVA: $F_{1,28} = 1.27$; P = 0.27), so we assumed that model size did not affect lizard aggressive response.

The videos were analysed in the platform BORIS (Behavioral Observation Research Interactive Software, Friard and Gamba, 2016, freely available at www.boris.unito.it). All the behaviours addressed to the stimulus were scored as follows: 1) no interest (i.e., walking across the terrarium without any interactions with the stimulus); 2) interest without aggression (i.e., observing or tongue-flicking towards the stimulus); 3) interest with overt aggression (i.e., biting against the stimulus).

Scores for the three treatments were compared using a Kruskal-Wallis test and Mann-Whitney tests were used as *post-hoc* tests. Analyses were performed under the R rel. 3.4.2 statistical environment (R Development Core Team, 2017) and, otherwise stated, reported values represent means and standard errors.

RESULTS

The higher aggression score (i.e., 3) was observed only for the mirror treatment, where 63.3% (19 out of 30) of males bit the stimulus. The highest score achieved by males in the other two treatments was 2, with 66.7% and 56.7% in control and silicone model treatments respectively. These differences were highly statistically significant (Kruskal-Wallis: $\chi^2 = 26.021$, d.f. = 2; P < 0.001). Mann-Whitney *post-hoc* tests showed that aggression scores did not differ between control and silicone models (P = 0.44); on the opposite, both comparisons involving

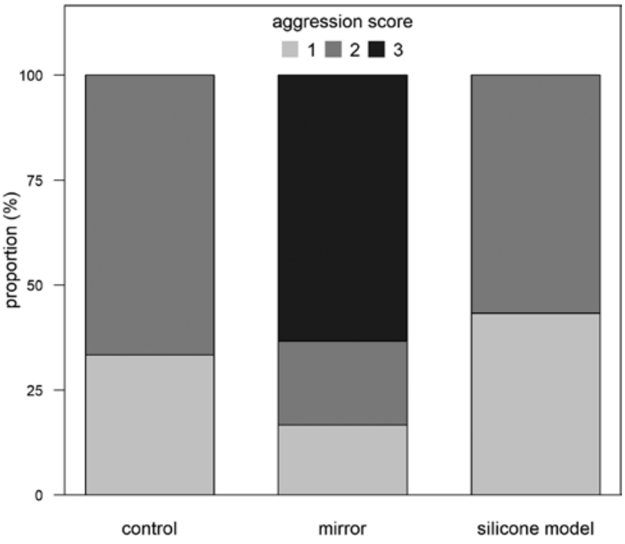

Fig. 1. Proportions of aggressive responses against the stimuli (control, plaster model, and mirror respectively) by common wall lizards (see methods for scoring details).

mirror were significant (P < 0.001 in both cases), being the score of the mirror treatment always higher than the other two (Fig. 1).

DISCUSSION

Our experiment demonstrated that visual stimuli are important releasers in triggering aggressive behaviours in the common wall lizard. It is often difficult to disentangle the effect of single key stimuli in a complex stimulus, but the approach used in this study allowed us to separate the effect of a simple cue, the shape of a lizard, by the composite effect of shape and motion. The result is not as trivial as it might seem, because some lizard species actively respond to simplified and motionless models by activating some territorial behaviours, demonstrating that they do not necessarily need more complex visual stimuli. This is true for some Agamidae, such as the tawny dragon lizard, *Ctenophorus decresii*, whose territorial behaviour has been intensively studied. Indeed, males of this polymorphic species engage in complex displays to defend territories and they use the same behavioural patterns also against models, responding differently even to throat colours (Yewers et al., 2016). Also cordylids, such as *Platysaurus minor* and *P. monotropis*, show overt aggression behaviours against models, demonstrating poor species recognition when models of different congenerics were proposed (Korner et al., 2000). All the above examples support the hypothesis that oversimplified visual stimuli can provoke an aggressive response in these taxa.

The common wall lizards were not interested in the presence of models within the enclosures and their only possible reaction was moving sometimes around and on the fake lizards and tongue-flicking at a certain distance. These behaviours were adopted even when the control stimulus (i.e., the white paper sheet) was inserted in the enclosure, so no conclusion can be inferred because they could be due to simple exploration activity. By contrast, the mirrored image always caused an alert posture, such as "freezing" in front of the mirror, repeated tongue-flicking or push-up displays, often culminating in overt aggression against the image with multiple bites or jumps. Numerous authors proved that mirrors are efficient stimuli able to release aggressive behaviours for many species belonging to different families (Agamidae, Phrynosomatidae, and Dactyloidae) (Korzan et al., 2000; Brandt, 2003; Farrell and Wilczynski, 2006; Norfolk et al., 2010; Osborne et al., 2012; Dunham and Wilczynski, 2014). Interestingly, in all these cases the species belong to visually-oriented lizard clades, making such a result expectable. On the opposite, to our knowledge this is the first time that the same kind of visual stimulus (i.e., a mirrored image) releases aggressive behaviour in a lacertid lizard, which is thought to be more chemical- than visual-oriented (but see Garcia-Roa et al., 2017; Baeckens et al., 2018). One main objection to the reliability of our results could be the different smell between the proposed stimuli and a real lizard, but we chose to exclude chemical stimuli in our experiment to disentangle the effect of shape, movement, and chemical hints. Our results confirm the observations by other authors that aggression releasers often are not single visual stimuli, such as a still image, but the combination of different stimuli, such as shape and motion (Macedonia and Stamps, 1994; Yang et al., 2001; Ord et al., 2002; Yang and Wilczynski, 2002; Van Dyk and Evans, 2008; Frohnwieser et al., 2017).

Previous research on the role of motion patterns in the visual displays of anoles demonstrated that motion is fundamental to attract the attention of lizards, particularly when a specific motion pattern is exhibited (Fleishman, 1992). The response to motion patterns is used by many territorial species to signal their presence to intraspecific opponents. These lizards use dewlap extension to communicate with rivals and partners and this display is often accompanied by a stereotypical head-bobbing movement. A detailed study on signal efficacy showed that motion patterns that combined high acceleration with high velocity were particularly effective. Nonetheless, at a short distance, even a small-amplitude motion in the visual periphery can be perceived by a lizard, causing a shift of gaze so that the image falls on a high-resolution portion of the retina (Fleishman, 1992). Of course, lacertids and anoles do not share the same evolutionary history (Pyron et al., 2013), so a detailed study on visual acuity and efficacy of the formers would be hoped. Simple stimulus, such as shape, could be enough to trigger a territorial response in basal lizard clades, but not in modern lizards that use chemical stimuli as the main releaser in intraspecific communication. Previous studies on animal communication demonstrated that the sensory system of the receiver determines which signals can be detected and that, in majority of cases, sensory systems serve multiple purposes and must be capable of detecting many types of different stimuli, such as mates, food, habitat, and opponents (Fuller and Endler, 2018). Responding to all the stimuli could be extremely costly, so there is selection on sensory systems to efficiently capture relevant stimuli in the environment (Fleishman, 1992; Fuller and Endler, 2018). This could explain why motionless or slow-moving shapes do not elicit aggressive responses in the common wall lizard, whereas mirrored images do.

Further studies will be necessary to fully understand the aggressive behaviour of the common wall lizard and the underlying releasing mechanisms, as well as to support the relationship between phylogeny, territoriality, and the complexity of visual stimuli. Nevertheless, we demonstrated that mirrored images are able to activate the aggressive response also in a lacertid lizard, which sets the stage for a wider and comparative study using other species and stimuli.

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SUPPLEMENTARY MATERIAL

Supplementary material associated with this article can be found at < http://www.unipv.it/webshi/appendix > Manuscript number 24651.

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CHAPTER 2

Close encounters of the three morphs: does color affect aggression in a polymorphic lizard?

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RESEARCH ARTICLE

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Close encounters of the three morphs: Does color affect aggression in a polymorphic lizard?

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Abstract

Color polymorphism is genetically controlled, and the process generating and maintaining morphs can affect speciation/extinction rates. Color badges are useful signals in intraspecific communication because they convey information about alternative strategies and can potentially decrease unnecessary conflicts among different color morphs. Competition and aggressive interactions among color morphs can contribute to polymorphism maintenance. This could lead to an uneven spatial distribution of morphs in a population because the local frequency of each morph establishes the intensity of the competition and the fitness of each male. We used a polymorphic lizard, *Podarcis muralis*, to assess if aggression varies among morphs under two contrasting hypotheses: a heteromorphic versus homomorphic aggression. We used laboratory mirror tests after lizard color manipulation, and we verified the consistency of results with an analysis of the spatial distribution of morphs in a wild population. Both the experiments confirmed that aggression is more common during homomorphic than heteromorphic contests. The adoption of alternative behavioral strategies that minimize risks and costs could facilitate the stable coexistence of the phenotypes and reduce competition. A bias in aggression would advantage rarer morph, which would suffer less harassment by common morphs obtaining a fitness advantage. This process would be negatively-frequency-dependent and would stabilize polymorphism, possibly contributing to sympatric speciation.

KEYWORDS
alternative strategies, color polymorphism, homomorphic aggression, lacertid lizard, mirror test, morph spatial distribution, *Podarcis muralis*

1 | INTRODUCTION

Color polymorphism occurs when two or more heritable color morphs “coexist in temporary or permanent balance within a single interbreeding population [...] in such frequencies that the rarer cannot be due solely to mutation” (Huxley, 1955). Morphs are genetically controlled and can evolve by both natural and sexual selection, and the process generating and maintaining it can affect speciation rates and/or extinction rates, either positively or negatively (Gray & McKinnon, 2007; Hugall & Stuart-Fox, 2012).

In several species of polymorphic lizards, morphs generally associate with alternative reproductive strategies, modulated by complex interactions among environmental pressures (e.g., social interactions and individual density), and therefore represent locally adapted optima (Roulin, 2004; Sacchi et al., 2007; Sinervo & Lively, 1996; E. Svensson et al., 2001). When males of different morphs associate with opposite breeding strategies, the intensity of the aggressive behavior may vary among morphs. Color may predict the outcome of a dyadic encounter, irrespective of other asymmetries in size, residency, or prior experience (Hover, 1985; Sinervo & Lively, 1996; Thompson & Moore, 1991a, 1991b). For example, green males of the tree lizard (*Urosaurus ornatus*) are more likely to dominate orange ones despite their lower size (Hover, 1985); orange-throated males of side-blotched lizards (*Uta stansburiana*) are highly aggressive and dominant over the other morphs (Sinervo & Lively, 1996), as well as red males of the Australian painted dragon (*Ctenophorus pictus*) are more likely to win dyadic contests with yellow males (Healey et al., 2007). However, in other polymorphic lizard species, the role of color morph is often ineffective at predicting the outcome of male-male contests (Sacchi et al., 2009; Stuart-Fox & Johnston, 2005). For example, color morph does not predict the contest outcome or aggression levels for two species of dragon lizards, even if a marginal effect may subsist only during fights between unfamiliar opponents (Stuart-Fox & Johnston, 2005). Such discrepancies can be explained by the occurrence of multiple mixed signals, so that color badges could not be the only way lizards use to communicate their fighting abilities. In fact, many other signals such as posture, movements, or chemical secretions can mediate the opportunity of engaging in physical combats (e.g., Martin & López, 2015; Van Dyk & Evans, 2008). Furthermore, prior antagonistic experience could override color importance in contests because a defined hierarchy can be established between individuals, avoiding unnecessary combats (Stuart-Fox & Johnston, 2005).

One of the mechanisms that can help to maintain variation in a population is negative frequency-dependent selection resulting from some processes, such as predation (Olendorf et al., 2006; Qvarnström et al., 2012), sexual selection (Sinervo & Lively, 1996), and intraspecific competition (Seehausen & Schluter, 2004), that guarantee an advantage to rare morphs. Morphs could reduce intraspecific competition by divergence in resource use (i.e. a character displacement). This process is acknowledged as one of the possible mechanisms for sympatric speciation (Qvarnström et al., 2012; Svensson, 2017) and makes the study of color polymorphism particularly attractive for evolutionary researchers. Aggression biases evolved to avoid unnecessary conflict among morphs, but in some cases, morphs adopt alternative strategies that are an outcome of male-male competition and maintain genetic variation and enhance the reproductive success of each morph under context-dependent control (Bleay et al., 2007; Hurtado-Gonzales & Uy, 2010). In this scenario, males of rare morphs should receive less aggression from other males because they do not share the same resources, and thereby they should experience a fitness advantage (Qvarnström et al., 2012; Seehausen & Schluter, 2004). The spatial scale at which behavioral interactions among morphs confer a fitness benefit is the composition of morph within a neighborhood because the local frequency of each morph establishes the intensity of the competition in that neighborhood, and consequently the fitness of each male (Zamudio & Sinervo, 2003). When morphs associate with alternative breeding strategies, the fitness of a given male depends on the number of males that come in direct competition with him, and consequently on the relative frequency of morphs in its neighborhood. For example, the three male morphs of the side-blotched lizard (*Uta stansburiana*) exhibit a combination of alternative breeding strategies that interact in a cyclical dominance hierarchy, where A > B, B > C, C > A (Sinervo & Lively, 1996). Orange males are aggressive and defend a territory, blue-males are aggressive but are mate-guarder, and yellow males are not aggressive and behave like sneakers (i.e., opportunistic individuals that do not own a defined territory or partner and mate with females occurring in other lizards' territories). Each morph has specific behavioral traits that allow it to outcompete only one of the other two. So, orange males outcompete yellow males but are defeated by blue males. There is experimental evidence that the frequency-distribution of morphs in a neighborhood of each male directly affects its fitness, in a way that orange males in a neighborhood of blue males achieve much less fitness than orange males in a neighborhood of yellow males (Zamudio & Sinervo, 2003). Therefore, the analysis of the spatial distribution of morph at the neighborhood scale can reveal information on the competitive interactions among the strategies behind morphs. In both cases (i.e., aggression bias or trade-off of alternative strategies), negative-frequency dependent selection, deriving from the evolved differential aggression, could lead to rare-male advantage and maintain polymorphism,

Furthermore, morphs could reduce intraspecific competition by divergence in resource use (i.e., a character displacement) and this process is acknowledged as one of the possible mechanisms for sympatric speciation (Qvarnström et al., 2012; Svensson, 2017) and makes the study of color polymorphism particularly attractive for evolutionary researchers.

The common wall lizard (*Podarcis muralis*) is a small (50–70 mm adult snout–vent length, SVL) diurnal lizard of central and south-eastern Europe (Sillero et al., 2014), whose males vigorously fight for territories, showing a marked territorial behavior (Edsman, 1990; Sacchi et al., 2009). Previous studies demonstrated that this species expresses a pigment-based ventral color polymorphism in both sexes with three discrete color morphs (white, yellow, and red; Sacchi et al., 2013; Sacchi et al., 2007). Morphs are genetically determined (Andrade et al., 2019) and correlations between morphs and aggressiveness remain controversial (Abalos et al., 2016; Coladonato et al., 2020; Sacchi et al., 2009). In a previous study using a resident-intruder design, Sacchi et al. (2009) showed that simple rules, such as residency and body size differences, determine the outcome of agonistic encounters but the authors did not find any effect of color on male aggression or fighting success. By analyzing dyadic encounters in a neutral arena, Abalos et al. (2016) found that the black patches on ventral ornamentation emerged as a good predictor of contest outcome independently of morphs, even if red males lost fights against heteromorphic males more often than yellow or white males. However, this effect could be due to a correlation with the size of black patches. The plasmatic concentration of testosterone also did not differ among morphs (Sacchi et al., 2017). However, it varied seasonally, as yellow males maintained significantly higher T-levels over time and displayed a stronger subsequent decline. The hormone profile did not differ between red and white males (Sacchi et al., 2017). Accordingly to seasonal variation in hormone profile, Coladonato et al. (2020) were able to detect morph-specific differences in the seasonal pattern of variation in the aggressive behavior of yellow males compared to males of the white morphs, but not among white, yellow, and red males. Overall, no clear picture emerges from these studies, so a study able to prevent any confounding effect given by staged contest is needed to fully understand the role of visual badges in releasing aggressive behaviors and the strategies adopted by each morph.

A recent study highlighted the effectiveness of mirror experiments for measuring the intensity of the aggressive behavior in lizards, demonstrating that they do not show self-recognition and attack the mirror image as a true "rival" (Scali et al., 2019). Mirror tests have the great advantage of allowing the experimenters to control for the effect of asymmetries in size and residence/motivation, as well as color signal since each individual can be acclimated in one cage until it becomes resident before facing an intruder having the same size and giving positive feedback during aggressive contests (Scherer et al., 2016). In this study, we used mirrors to assess if aggression varies among morph after removing the main determinants of contest outcome in this species (i.e., size and residency, Sacchi et al., 2009). In detail, we explored two contrasting hypotheses: (1) a heteromorphic aggression hypothesis (three morphs hypothesis, hereinafter 3MH), where each morph compete with others to verify if some morphs are more aggressive than others or if some morphs are more aggressive towards a specific morph; (2) a homomorphic aggression hypothesis (hereinafter HH), where each morph shows a higher aggression level when faced with an opponent displaying the same color (i.e., the same strategy). Even if the two hypotheses are not mutually exclusive, we chose to test both because the homomorphic one could be masked by the heteromorphic one in a three-level comparison. Furthermore, there is no evidence of a cyclic or intransitive system (sensu Permogorskiĭ, 2014; Sinervo & Lively, 1996), nor a hierarchical one in the common wall lizard's polymorphism, so no a priori assumptions could be made about which morph dominates which one. In any case, our experiment could shed new light on the competitive interactions among morphs of this species. These hypotheses were tested in two ways (1) a laboratory experiment where we used mirrors to test aggression after color manipulation, and (2) an intensive field sampling where we assessed if the spatial distribution at neighborhood level of different morphs supports the results of the laboratory experiment.

2 | METHODS

2.1 | Laboratory methods

Thirty-six adult male lizards (12 for each pure morph) were captured by noosing during the reproductive period (April–June 2018) in five sites in the Milan province (northern Italy). Animals were carried to the Natural History Museum of Milan and housed in individual plexiglass boxes (40 × 40 × 30 cm) with a refuge (a hollow brick) positioned near one box's wall, water ad libitum, and fed with three mealworms (*Tenebrio molitor*) larvae/day. A sheet of absorbent paper was used as substrate so that the terrarium could keep the resident odor and the lizards could consider it as their territory. The vertical sides of the boxes were covered externally with white paper sheets, to avoid external stimuli and wall reflectance.

After three days, lizards were tested in the same terrarium where they were acclimated, removing water and food. The ventral color was manipulated painting throat and belly with water-based tempera colors, randomly assigning all the color combinations with four replicas for each combination. This means that the twelve lizards from each morph were divided into three subsamples of four individuals, and each subsample was treated with one of the three colors (white, yellow, and red). Each individual was tested for four consecutive days, maintaining the same coloration used in the first treatment, to verify response consistency. A total of 144 trials were performed during the experiment (12 individuals × 3 morphs × 4 trials).

A heating lamp (ZooMed Repti Basking spot lamp, 150 W) was turned on for 15 min before each trial to achieve an optimal body temperature similar for all the individuals (Sannolo et al., 2014). A photographic set was then placed on the terrarium providing uniform led lightning and a webcam (Microsoft Life Cam HD-3000) was used to record the trial. The lizard was put under the refuge for one minute while the mirror (30 × 15 cm) was positioned at the opposite side of the terrarium. When the camera was turned on, the refuge was removed and the behaviors were recorded for 15 min. All the lizards were released, healthy, at the capture sites after the experiments.

The videos were analyzed using BORIS (Behavioral Observation Research Interactive Software), an open-source software (www.boris.unito.it, Friard & Gamba, 2016). We counted the number of bites against the mirrored image to quantify the aggressive response to the stimulus. Since some lizards never bit the mirrored image, to prevent zero-inflation problems, the number of bites was transformed to the probability of bites during the trial (BP = no. of interactions with bites/no. of total interactions). We defined "interactions" any time the lizard entered the half of the terrarium bearing the mirror. Individuals who never interacted with the mirror in any trial were discarded from the following analyses. Some other displays were observed during the experiments (e.g., tongue-flicking, hand-shaking, tail-shaking, etc.), but we only considered those ones that could be interpreted as aggressive interactions toward the opponent and not those related to stress or explorative behavior (Coladonato et al., 2020; Sacchi et al., unpublished data).

2.2 | Field methods

Field sampling was performed in the archeological site of Castelseprio (Province of Varese; UTM 32 T 489077E, 5063874 N; 338 m a.s.l.). The field site is not the same as the collection sites used for the lab experiment, but they are located within a 30 km range, within a species distribution continuum, so we are confident that no significant differences among populations occur as far as behavior is concerned. The site is on the top of a hill and is characterized by stone ruins in an open area with grass or bare soil surrounded by natural deciduous woods. The study area is 2380 m^2 wide, and ruins cover about 10% of the whole surface and their height ranges from 30 cm to 5 m. Common wall lizards are scattered on the ruins and along the wood borders of the whole study area. Field sampling was performed by ten researchers on 3rd April 2017, (09:00–18:00), during lizards' reproductive period, intensively searching across the whole area. We restricted the sampling to a single day to have a stationary picture of the distribution of lizards in the study area. We captured a total of 255 lizards by noosing, including 206 adults lizards (SVL > 56 mm, Sacchi et al., 2007). Lizards were marked with a nontoxic color to avoid pseudoreplication and released after recording their sex, morph, and position. To be sure that the position errors were small enough, compared to inter-lizards distances (e.g., less than 10 cm), lizards were located on a high-resolution orthoimage obtained by the use of a Remotely Piloted Aircraft System (Figure 1; Supporting Information Material): the map had mean horizontal and vertical errors of 3.5 and 2.2 cm, respectively. Analyses were performed on 144 adult males, excluding females, juveniles, and three males (yellow-red) that cannot be unambiguously assigned to a single morph.

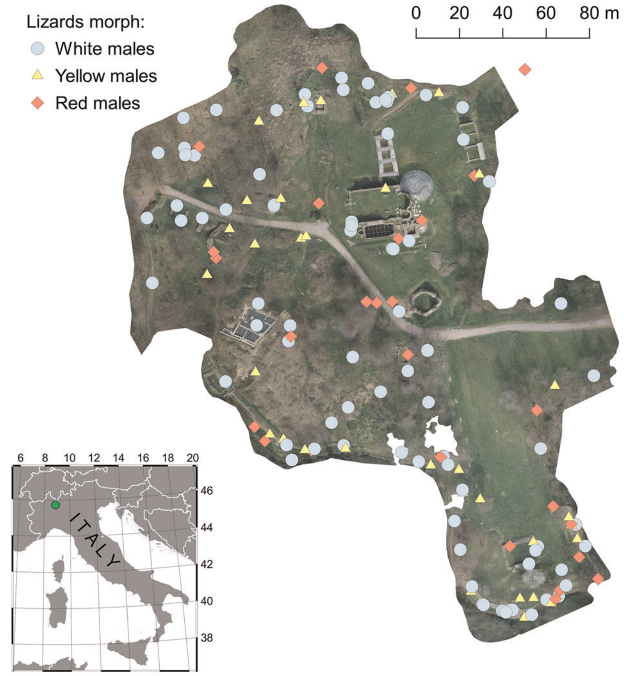

FIGURE 1 Position of the 144 adult males considered in the analyses. Orthoimage was obtained by the use of a Remotely Piloted Aircraft System, as described in the Supporting Information Material (Menegoni et al., 2019) [Color figure can be viewed at wileyonlinelibrary.com]

2.3 | Ethical statement

This study was carried out in conformity with current European and Italian laws on animal use in scientific research (authorized by the "Ministero dell'Università e della Ricerca," MIUR, prot. 0002154/PNM, 2016, 2nd March, valid over 2016–2018) and Italian laws for the access to the Archeological Sites (authorized by the "Ministero dei Beni e delle Attività Culturali e del Turismo", prot. n. 1441/MIBACT-SAR-LOM, 2017, 17th March). All the experiments were performed in accordance with national guidelines for the care and use of animals in laboratories and all the animals captured for laboratory and field experiments were released healthy at the capture site.

2.4 | Laboratory statistical analyses

Laboratory data were analyzed using a generalized linear mixed (GLMM) model with a binomial error distribution, using bite probability (BP) as the response variable. For the 3MH hypothesis, we used a model with morph (three-levels factor: W, Y, R), treatment (three-levels factor: W, Y, R), and their interaction as fixed factors, trial (entering as a continuous variable given the constant time between subsequent trials) as covariate controlling for a potential habituation effect (Peeke, 1984); the lizard ID was used as a random factor on the intercept to account for repeated trials. For the HH hypothesis, we used a model with treatment (recoded into a two-level factor: homomorphic, heteromorphic) as the fixed factor, trial as the control covariate, and ID as the random intercept.

While the 3MH model tests both for intransitivity or hierarchy and for homomorphic versus heteromorphic hypotheses, the HH model only tests for homomorphic versus heteromorphic aggression. In the latter case, the statistical power is higher due to data pooling.

Predictor significance was evaluated by likelihood-ratio (LR) tests (Zuur et al., 2009). Analyses were performed under the R 3.45.2 statistical environment (R Development Core Team, 2018), using the package "glmmTMB" (Brooks et al., 2017).

2.5 | Field statistical methods

The same hypotheses (3MH and HH) used for the lab experiment were tested on field data. First, we mapped the distribution of all males (Figure 1) and computed for each focal lizard the distance to the nearest white (d_W), the nearest yellow (d_Y), the nearest red (d_R), the nearest homomorphic (d_{Ho}), and the nearest heteromorphic (d_{He}) conspecific. Secondly, we computed the differences in the minimum distance for each morph pair ($\Delta_{RW} = d_R - d_W$; $\Delta_{YW} = d_Y - d_W$; $\Delta_{RY} = d_R - d_Y$) associated to each focal lizard, and between the homomorphic and heteromorphic minimum distances ($\Delta_{HH} = d_{Ho} - d_{He}$): this way, we obtained a measure of the relative proximity of males of the three morphs, or of homomorphic and heteromorphic males to each focal lizard. In the end, we averaged these Δs according to the hypothesis to be tested: under 3MH, for each focal color morph we derived the three average, $\acute{\Delta}_{RW}$, $\acute{\Delta}_{YW}$, $\acute{\Delta}_{RY}$; under HH, the average $\acute{\Delta}_{HH}$. The departure of the average Δs from zero would indicate nonrandom distribution (repulsion or attraction among certain color morphs). However, since the number of individuals of the three morphs was not balanced (white = 81, yellow = 36, red = 27), differences in $\acute{\Delta}s$ may simply reflect the relative abundance of a given morph (i.e. a rarer morph can show a higher distance just by chance). This is true also for $\acute{\Delta}_{HH}$: even in a scenario where morphs relative abundance was balanced (1/3 white, 1/3 yellow, 1/3 red), the probability of being closer to a heteromorphic would be higher just because of chance. To account for this spatial bias, we adopted a data permutation procedure, and built the expected average $\acute{\Delta}s$ null distribution given the observed point pattern and assuming color morph not to affect the spatial distribution of male lizards. We first permuted lizard color morph (no. of permutations = 999), and we recomputed all the distances (d) and distance differences (Δ). We thus obtained the null distributions of all the average $\acute{\Delta}$ s for each focal color morph (3MH hypothesis), and the null distribution of $\acute{\Delta}_{HH}$(HH hypothesis). We then assessed the probability of each observed $\acute{\Delta}s$ being larger or smaller than the respective null distribution (depending on the observed value being larger or smaller than the mean null value). Even in this case, the statistical power of the 3MH model is lower than the HH model due to data pooling in the latter test.

3 | RESULTS

3.1 | Laboratory experiment

Out of the 36 lizards tested, six males never responded to the stimulus, that is, they never moved after refuge removal or they never enter the half of the terrarium bearing the mirror. They were considered as failed trials and excluded from the analysis. From the remnant 30 focal lizards, we obtained 119 usable trials; one was excluded because the focal lizard did not enter the half cage with the mirror. So, 29 lizards had four replicas, and one had three.

The 3MH model for BP did not reveal any significant main effect (morph: LR, $\chi^2 = 0.359$, d.f. = 2; $p = .836$; treatment: LR, $\chi^2 = 1.539$, d.f. = 2; $p = .463$; morph × treatment: LR, $\chi^2 = 5.295$, d.f. = 4, $p = .258$; trial: $\chi^2 = 3.650$, d.f. = 2; $p = .056$). The effect of the trial number was borderline significant, with a weak negative effect on BP ($\beta_{estimate} = -0.170$; $\beta_{SE} = 0.089$). On the opposite, the random effect of the individual identity was highly significant (LR, $\chi^2 = 75.018$, d.f. = 5; $p < .001$), and accounted for 18.34% of the total BP variance, highlighting the occurrence of a strong variation among individual propensity to bite the mirrored image.

Considering the HH model, the trial was confirmed almost significant (LR, $\chi^2 = 3.656$, d.f. = 1; $p = .056$) and with an identical coefficient estimation ($\beta_{estimate} = -0.170$; $\beta_{SE} = 0.089$) as 3MH model. The recoded two-level treatment showed a significant effect, instead (LR, $\chi^2 = 5.584$, d.f. = 1, $p = .0181$), which highlighted a higher aggression level during homomorphic contests compared to heteromorphic ones (Figure 2). Again, the individual identity was highly significant

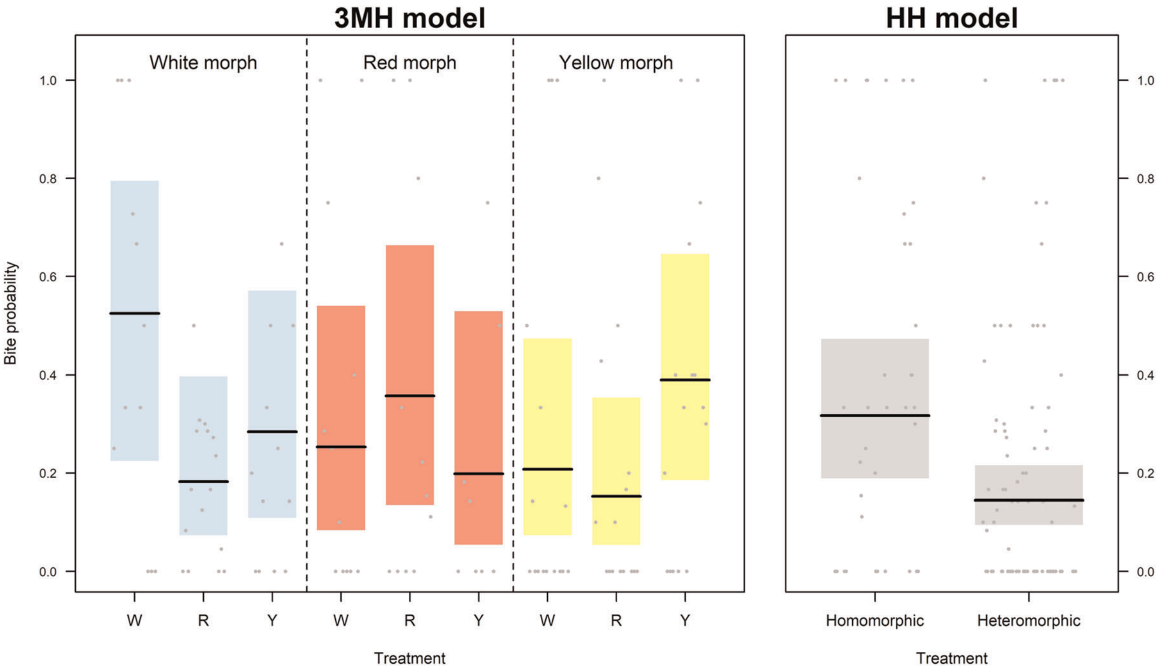

FIGURE 2 Effect of treatment and morph on the biting probability (BP), according to the heteromorphic aggression model (3MH, left panel), and on the homomorphic aggression model (HH, right panel). For the 3MH model, all morph × treatment combinations were shown. Gray points = observed values; horizontal thick lines = predicted BP values; color or gray shaded area = 95% confidence interval of predictions [Color figure can be viewed at wileyonlinelibrary.com]

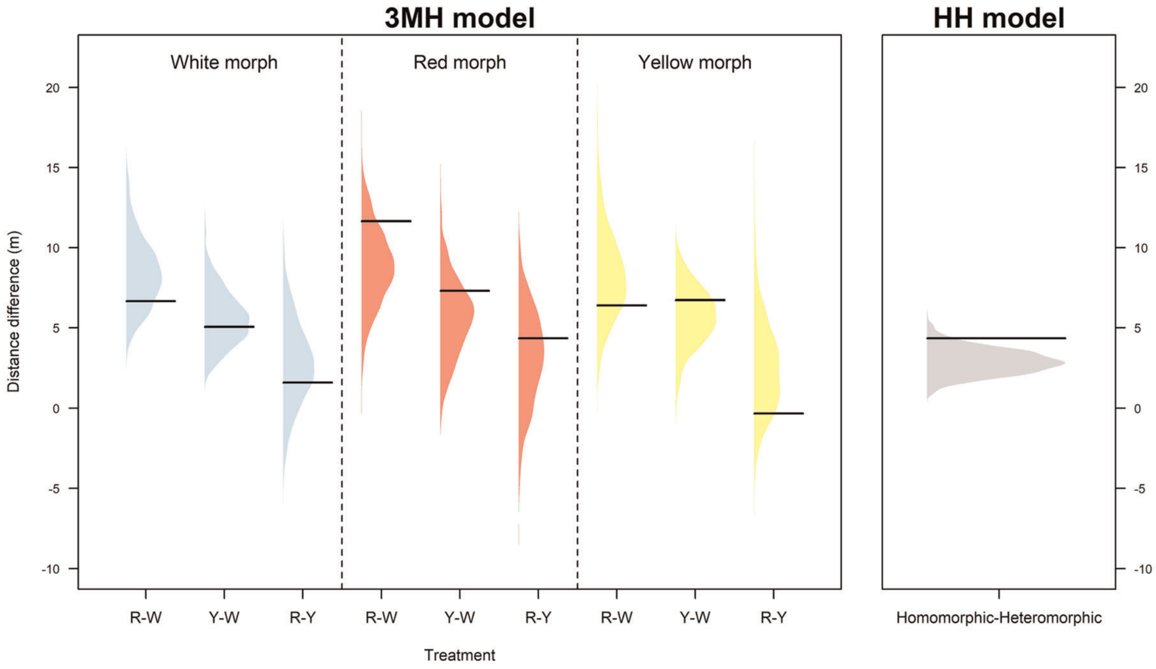

FIGURE 3 Observed distance differences (black lines) compared to the distribution of the simulated distance differences (color or gray shaded areas). Left panel: 3MH hypothesis; for each focal morph, RW represents the average distance differences between the nearest red male and the nearest white male; the same for the pairs YW and RY. Right panel: HH hypothesis; average distance differences between homomorphic and heteromorphic individuals [Color figure can be viewed at wileyonlinelibrary.com]

(LR, $\chi^2 = 43.367$, d.f. = 1; $p < .001$) and explained 19.80% of the total variance.

3.2 | Field experiment

No difference was observed in the mean distance difference between individuals when morph pairs were compared (Figure 3). For the white morph the observed mean distance differences were: $\Delta_{RW} =$ 6.66 m ($p = .229$); $\Delta_{YW} = 5.06$ m ($p = .398$); $\Delta_{RY} = 1.60$ m ($p = .317$). For the red morph, the observed mean distance differences were: $\Delta_{RW} = 11.66$ m ($p = .126$); $\Delta_{YW} = 7.31$ m ($p = .235$); $\Delta_{RY} = 4.35$ m ($p = .363$). For the yellow morph the observed mean distance differences were: $\Delta_{RW} = 6.39$ m ($p = .242$); $\Delta_{YW} = 6.73$ m ($p = .308$); $\Delta_{RY} = -0.33$ m ($p = .154$). The mean distance difference between homomorphic and heteromorphic individuals resulted instead significantly higher than the null mean distance difference ($\Delta_{HH} = 4.35$ m; $p = .041$; Figure 3).

4 | DISCUSSION

Our experiment demonstrated that the common wall lizard shows morph-specific aggressiveness. Males of this species are often aggressive toward conspecifics and this was demonstrated by the three morphs lab experiment, where almost all the individuals performed at least one aggression towards their mirrored image. Only six individuals never interacted with the mirror nor entered the half cage near the stimulus. This could be due to stress, fear, or personality since individual responses can differ according to the provided stimulus (static models, regular mirrors, nonreversing mirrors, or real opponents; Li et al., 2018; Sacchi et al., 2021). The comparison of the number of aggressive interactions between pairs of morphs did not reveal any prevalence in attacking a specific morph. However, when data were pooled and analyzed under the homomorphic versus heteromorphic hypothesis, significant aggression was observed toward individuals belonging to their same morph.

Morph-specific aggression has been studied in the past few years to understand mechanisms underlying polymorphism maintenance with contrasting results. Many studies were conducted on fish, mainly on cichlids, famous for their spectacular intraspecific diversity. In some cases, males compete more heavily with other males of the same color, as in our case study (Dijkstra et al., 2007, 2008, 2010; Seehausen & Schluter, 2004; van Doorn et al., 2004). In these cases, rare male phenotypes would receive less aggression than common male phenotypes, and this could generate frequency-dependent selection (Dijkstra et al., 2007; 2010; Qvarnström et al., 2012; Seehausen & Schluter, 2004). Although this mechanism does not contribute to the emergence of reproductive barriers, it can pave the way for sympatric speciation (Dijkstra et al., 2008).

A previous study demonstrated that common wall lizards can discriminate colors and can, consequently, recognize individuals belonging to their own morph (Pérez i de Lanuza et al., 2018). This ability presumably contributes to different results in staged contests, with red males having the lower fighting ability (Abalos et al., 2016), and to assortative mating, with homomorphic male–female pairs more common than the heteromorphic pairs (Pérez i de Lanuza et al., 2012). Assortative mating could be advantageous also in terms of fitness, since nonrandom mating produces clutches with different characteristics, in accordance with the r, K, and mixed strategies demonstrated for the common wall lizard (Galeotti et al., 2013). Under these assumptions, throat and belly coloration can be considered as a visual badge that common wall lizards use in intraspecific communication. Even if color polymorphism of common wall lizards occurs both in males and females, sexual selection seems to be driven mainly by male choice, whereas the importance of female choice is still debated (Sacchi et al., 2015, 2018). If males preferentially choose homomorphic females, then homomorphic males should be considered as the direct competitors for this resource, so the higher aggression level observed in our experiment can be explained as a need to defend the territories from intruders competing for the same potential mates (Seehausen & Schluter, 2004). This conclusion is supported by the lack of differences in aggression in the 3MH model. Similar results were obtained for the tawny crevice dragon (*Ctenophorus decresii*), an agamid with four discrete morphs (Teasdale et al., 2013), that showed a higher aggressive behavior during homomorphic contests when models were presented to free individuals (Yewers et al., 2016). The color perception of the opponent seems fundamental in triggering aggression also in other species, such as the ornate tree lizard (*Urosaurus ornatus*), as demonstrated by experiments where colors were manipulated (Hover, 1985).

Our field experiment supported these results, as the mean distance among individuals resulted significantly different when homomorphic and heteromorphic males were compared, in accordance with the HH model. In contrast, no difference was observed in the 3MH model, highlighting no variation in the mean distance between pairs belonging to the three morphs. This result suggests a nonrandom distribution of males in the study area. A similar result was observed in haplochromine cichlids of Lake Victoria, where breeding territories of individuals of different colors are closer than those of individuals of the same color (Dijkstra et al., 2006; Seehausen & Schluter, 2004). The uneven spatial distribution of male common wall lizards could be reinforced by the individual recognition given by scent marks, that contribute to give information about neighbors. In fact, in lacertids, femoral secretions convey information about neighbor characteristics, such as size, weight, and familiarity (Carazo et al., 2008). Proteins, in particular, give information about the identity and morph in *P. muralis* (Mangiacotti, Fumagalli, et al., 2019; Mangiacotti, Gaggiani, et al., 2019). Lacertid lizards are also able to remember the spatial location of scent marks (Carazo et al., 2008), so males could build a spatial map of neighboring rivals. In this way, they can decide which neighbors could exert a major threat to their territories and address aggression against their direct competitors, minimizing both the energetic costs of territory defense and the risks of suffering injuries or predation, according to the paradigm of the “dear-enemy effect” (Carazo et al., 2008; Tumulty, 2018; Whiting, 1999; Ydenberg, 1988).

We only tested the effect of a resident male towards an intruder male without analyzing the response of an intruder versus a resident lizard. In this case, other factors could intervene, such as motivation to defend an unknown territory. This hypothesis could be tested by inserting the focal lizards in unknown boxes, different from those used for acclimation. Indeed, a previous study with staged contests demonstrated that the motivation of resident individuals for territory defense could be a driving force to the contest outcome (Sacchi et al., 2021), so the aggressive response could be lowered by this condition and the role of color morph underestimated.

In conclusion, male aggression in the common wall lizard seems to be morph-dependent. The adoption of alternative behavioral strategies that minimize risks and costs of unwanted conflicts could facilitate the stable coexistence of the phenotypes (Dijkstra et al., 2006; Yewers et al., 2016). A bias in aggression to like-colored males would benefit the rarer morph, which would suffer less harassment by common morphs and obtain a fitness advantage. This process would be negative-frequency-dependent and would stabilize polymorphism in the populations (Dijkstra et al., 2006, 2008; Seehausen & Schluter, 2004).

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

Data will be uploaded on Zenodo, a free repository, only after final acceptance

PEER REVIEW

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SUPPORTING INFORMATION

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CHAPTER 3

Subjective resource value affects aggressive behavior independently of Resource-Holding-Potential and color morphs in male common wall lizard

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ARTICLE

Subjective resource value affects aggressive behavior independently of resource-holding-potential and color morphs in male common wall lizard

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Abstract

Game theory models predict the outcome of a dyadic contest to depend on opponents' asymmetries in three main traits: resource-holding potential (RHP), resource value (RV) and aggressiveness. Using male common wall lizards *Podarcis muralis*, a polymorphic species showing three discrete morphs (white, yellow, and red), we investigated how the aggressive behavior varies according to a change in subjective RV and color morphs, while controlling for the asymmetry in RHP (using mirrors). By comparing the aggression of the same individual towards its mirror image in two different arenas (familiar = high subjective RV; novel = low subjective RV), we showed that lizard aggressive behavior was more intense and prolonged in the familiar arena than in the new one, thus supporting the occurrence of a direct relationship between motivation and aggression in this species. We also found the overall aggressiveness to differ from individual to individual, supporting the general hypothesis that aggressiveness is a trait associated with personality. By contrast, no effect of morphs was detected, ruling out the occurrence of morph specific variation in the aggressiveness. Our results highlight that an individual's motivation and personality might be as important as RHP and RV in the resolution of animal contests.

Keywords Dyadic contests · Fighting rules · Aggressiveness · Personality · RHP · Motivation

Introduction

Agonistic encounters in animals occur when two individuals compete for a limited resource, such as food, territories or mates (Huntingford and Turner 1987; Archer 1988; Hack 1997). Contests carry costs for opponents, which may include an increased risk of injuries and mortality (Dufty 1989; Marler and Moore 1988), or predation risk (Lange and Leimar 2001; Tuttle and Ryan 1981), in addition to the costs of losing the disputed resource. Natural selection should favor the evolution of behaviors (i.e., strategies) and correlated phenotypes that minimize those costs, thus maximizing the probability of obtaining the disputed resources (Maynard-Smith and Price 1973). This in turn has favored the evolution in some species of fighting rules that normally solve intraspecific disputes without physical combat (e.g., Huntingford and Turner 1987; Jablonski and Matyjasiak 1997; López and Martín 2001), whereby interactions escalate to a physical fight only when it is not possible for the two contestants to reliably assess their respective fighting abilities (Sacchi et al. 2009).

Game theory models have shown that three main asymmetries between opponents can predict the outcome of a dyadic contest (Maynard-Smith 1982). The most common is the asymmetry in the ability to gain and hold the disputed resource (i.e., the "resource-holding potential", RHP, Parker 1974), which usually depends on body size and weapons (Caldwell and Dingle 1979; Sneddon et al. 1997). The individual with the higher RHP typically goes on to win (Parker

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1974; Maynard-Smith 1982; Elias et al. 2008; Arnott and Elwood 2009), and the more similar the opponents' RHP, the greater the probability that encounters will escalate to physical combat (Sacchi et al. 2009).

The second asymmetry affecting the contest outcome is the value for resources (RV), such as mates, territories, food or shelter (Parker 1974; Maynard-Smith and Parker 1976). In general, when the outcome of contests cannot be predicted from differences in RHPs, the individual defending a resource with higher RV is more likely to win (Parker 1974; Maynard-Smith 1982; Kokko 2013). Examples of how RV can influence fight outcome are seen in mammals (Barnard and Brown 1982), birds (Ewald 1985), amphibians (Verrell 1986), fishes (Lindström 1992), and invertebrates (Gherardi 2006; Brown et al. 2007). In some cases, a positive asymmetry in RV can compensate for negative asymmetry in RHP, as occurring in resident vs. intruder dynamics (Sacchi et al. 2009).

RV depends on both external factors, i.e., detectable properties of the resource that can be assessed by the contestants, and internal factors, i.e., the value that the individual contestants put on that type of resource (Gherardi 2006; Goubault et al. 2007; Mathiron et al. 2018). The first is often reported as the "objective RV", whereas the second one as the "subjective RV" (Gherardi 2006; Mathiron et al. 2018). The objective RV specifically relies on the fitness value intrinsic to the resource, and is dependent on its detectable properties (e.g. food, nesting sites, microclimatic conditions, refuges within a territory). By contrast, the subjective RV reflects the value that the two opponents place on that resource, and depends on the opponents having prior knowledge of the resource, as well as their own physical condition at the time of the contest. The interaction between objective and subjective RVs establishes the level of asymmetry in RV between opponents. For example, territories have their own values in term of fitness (objective RV), but residents may have a higher subjective RV than intruders due to their prior knowledge about territory properties. More generally, resource owners have higher RV than intruders (Gherardi 2006). Examples come from contests over nesting burrows in female iguanas (Rand and Rand 1976) or over females in male spiders (Austad 1983). Similarly, RV may increase following previous investment in the resource by the owner (Tobias 1997), as well as wins in previous encounters (i.e. the winner–loser effect, Mesterton-Gibbons and Dugatkin 1995; Hsu et al. 2006).

The third asymmetry concerns aggressiveness, as the individual tendency to escalate a contest independently of RHP and RV (Barlow et al. 1986; Maynard-Smith and Harper 1988). In this respect, aggressiveness differs from RHP and RV because it is an inherent property of the individual, a persistent trait of its personality, rather than a variable motivational state (Barlow et al. 1986; Santostefano et al. 2016). In comparison to RHP and RV, aggressiveness has received much less attention, despite its importance in determining an individual's fitness ability (but see Keeley and Grant 1993; Stienecker et al. 2019). For example, Barlow et al. (1986) and Keeley and Grant (1993) clearly demonstrated that cichlids become more aggressive when RV increases, therefore increasing an individual's probability of winning the contest.

Lizards have been extensively used for testing hypotheses about the effects of asymmetries in RHP (e.g., Molina-Borja et al. 1998; Sacchi et al. 2009; McLean and Stuart-Fox 2015; Quintana and Gladino 2017) and RV (Tokarz 1985; López and Martín 2001) on contest outcome. Often residency and body size predict outcomes: resource owners (i.e., residents) are more aggressive than intruders, and larger males are competitively superior to smaller ones (Molina-Borja et al. 1998; Sacchi et al. 2009; McLean and Stuart-Fox 2015; Quintana and Gladino 2017). Conversely, the roles of individual aggressiveness and motivation on contest outcome have been rarely investigated (but see Rangel-Patiño et al. 2018). This is likely due to the difficulty of controlling asymmetries in RHP, including body size and condition, between opponents. In a recent study (Scali et al. 2019), we showed that lizards consistently display an aggressive response to their own mirror image. This method can be effectively used to control asymmetries in RHP and RV between opponents when measuring contest behaviour.

In the present study, we investigated contest behavior in the common wall lizard (*Podarcis muralis*), a small (50–70 mm in adult snout–vent length, SVL) diurnal lizard of central and southeastern Europe (Sillero et al. 2014). In this species, body size and residence affect the outcome of dyadic encounters for territory ownership (Sacchi et al. 2009). Individuals of both sexes exhibit three discrete color morphs (white, yellow, and red) (Sacchi et al. 2007), which are genetically determined (Andrade et al. 2019). Correlations among morphs and aggressiveness are controversial (Sacchi et al. 2009; Abalos et al. 2016; Coladonato et al. 2020); in our previous study using a resident-intruder design (Sacchi et al. 2009), color morph did not affect the encounter outcome, but Abalos et al. (2016) were able to detect morph specific effects by analyzing dyadic encounters in a neutral arena.

We used mirrors to measure the aggressive response of a lizard in response to changes in subjective RV, accounting for potential variability in aggression between color morphs. Lizards were housed in two arenas with low and high subjective RVs, and we compared an individual's aggressive response to its mirror image. Our main prediction was that lizards would modulate their aggression according to the RV of the arena.

Methods

Lizard collection and housing

Sixty adult male common wall lizards (SVL range: 56–70 mm) were captured during spring 2018 (5th April–6th June) in and around the town of Pavia (Northern Italy, 45°11′N, 9°9′E): 36 were captured from three urban gardens in the town, and 24 were from three rural properties. Only pure morphs were collected, representing white ($n = 22$), yellow ($n = 22$), and red ($n = 16$), according to Sacchi et al. (2013). We individually housed lizards in 20 × 30 × 20 cm transparent plastic enclosures (Baeckens et al. 2016; Mangiacotti et al. 2019), with a flat brick as shelter/basking site and a small bowl of water. We provided mealworms as food (one mealworm/day). The housing room was maintained between 15 and 30 °C (the natural temperature range for the season), and large windows provided natural daylight. A minimum acclimation period of one week was given before starting trials, and we released all lizards at their capture sites following use in trials no more than 2 weeks after their capture date. All enclosures were carefully cleaned before a new individual was placed into it to remove any chemical cue of a previous subject. No lizard was injured or killed during the study, and all lizards looked healthy at release.

Experimental setting

A mirror was used to simulate the intrusion of a rival male within the enclosure and measure the male's aggressive response. We had previously shown that common wall lizards perceive their own mirror image as a rival, and behave aggressively in response, sometimes even biting (Scali et al. 2019). This method has the main advantage of allowing the experimenters to control for the effects of differences in size and motivation between opponents on the aggressive response of the focal male since the mirror image exhibits the same size and motivation of the male in this study. We measured the aggressive response of the same individual in two different contexts by the introduction of a small mirror (15 × 15 cm) into the arena. The first experimental treatment was conducted in the enclosure where the lizard had been acclimated, which was assumed to be a familiar context within its own territory (hereafter, "home" treatment). The second treatment was conducted in a new clean enclosure with the same size and setting as that of the familiar home treatment, but into which the lizard was moved only 15 min before the trial. This second treatment was assumed to be an unfamiliar context outside its own territory (hereafter, "neutral" treatment). Before starting the trial, we first put a partition dividing the arena into two halves, and placed the mirror at the far end of the half without the lizard. After a 5-min period to allow the lizard to habituate to disturbance, we removed the partition, thereby allowing the lizard to interact with the mirror. The fact that the mirror reflects the same color morph of the male is not problematic. In a parallel study involving the manipulation of throat color, we found that males deliver the highest magnitude response towards their mirror image when painted with their own color, and no significant difference occurred in the peak response among morphs (Scali et al. 2020). To avoid visual disturbance during the trials, the four sides of the arena were externally covered by opaque, white plastic panels. Before each trial, the male was heated for two minutes using a 75 W halogen infra-red lamp (Reptiles-Planet.com) positioned 40 cm above the arena. After switching off the lamp, the mean (± SD) body temperature of males just before starting the trial (measured with a handheld infra-red thermometer Lafayette TRP-39, Lafayette Instrument Co., Lafayette, Indiana, USA; sensitivity: 0.1 °C; precision: ± 2%) was 38.4 ± 1.9 °C. After removing the partition, the movements of the lizard were recorded using a webcam (Microsoft LifeCam HD 3000) mounted on an easel, 60 cm above the arena, and connected to a laptop by a 3 m cable. Recording was managed by Free2X software v1.0.0.1 (freely available at: http://www.free2x.com/webcam-recorder/), setting quality to 800 × 600 pixels and 15 frames per second (fps). Recording duration was set to 20 min (18,000 frames) after the first movement of the male. Room temperature was set to 28 °C to reduce thermal loss during the trials. Trials took place between 10:00 and 14:00. Each lizard took part in two trials (home and neutral treatments) on two subsequent days (one trial per day), and we randomized the first treatment in a way that half of the males was initially tested with the home treatment, and half with the neutral treatment. We repeated a trial the subsequent day if the lizard did not move after 10 min from the start. Overall, we performed 127 trials between 24th April and 29th June, and on average (± SD) each individual was tested 2.1 ± 0.4 times (range 2–4).

Response variables

We used BORIS (Friard and Gamba 2016) to analyse video files and extract four response variables. We used the first three variables to assess aggressive behavior as (i) the time (seconds) spent in the half of the arena containing the mirror (Time), (ii) the number of times the lizard entered the half of the arena containing the mirror (Nmirror), and (iii) the total number of bites against the mirror image (Bites). The variables can be interpreted as increasing levels in a rank of aggression from a low/moderate interest for the mirror image (Time and Nmirror) up to the open aggression against it (i.e., Bites). In detail, Nmirror measures the frequency the male approaches the "rival", irrespective of the duration of the interaction. Time was used as a proxy for the intensity of those approaches (as a sort of level of threat of the interactions): the higher the Time, the lower the mean distance of the male from the opponent. The fourth variable was the number of tongue flicks (TF) measured in the half of the arena containing the mirror. TF are used by lizards to collect chemicals from the environment and can be regarded as a proxy for the interest of an individual for an external stimulus (Cooper 1991, 1994). TF is not related to aggressive displays (Greenberg 1993), and we used it as a control for the occurrence of any effect of the experimental treatment on the lizard's behaviours other than the aggressive response. For simplicity, we hereafter refer to Bites, Time and Nmirror as forms of "aggressive behavior", and to TF as "exploratory behavior". All response variables achieved normality (Bites required a log-transformation), and all showed low intercorrelations (Pearson correlation coefficient $|r_{\text{Pearson}}| < 0.54$).

Statistical analyses

To examine if lizards responded differently to the experimental treatment, we used random intercept linear mixed models (LMM), one for each response variable. Fixed effects were the treatment (home vs neutral), the morph, and the trial (first vs second) to account for the sequence of stimulation. All the two-way interactions were added to account for possible differential effect of treatment due to the sequence of the trials and morphs. We also added, as fixed effect, SVL, temperature and date (Julian date) to control for possible confounding effect due to individual size, temperature in the arena and season. These three variables were standardized by subtracting the mean and dividing by the standard deviation. The individual entered the model as random effect. LMMs were fit in a Bayesian analytical framework available in the package JAGS 4.3.0 (http://mcmc-jags.sourceforge.net/), using flat priors for coefficients and intercept ($\mu = 0$ and $\sigma = 0.001$), and uninformative half-Cauchy priors ($x_0 = 0$, $\gamma = 25$) for both σ^2_{error} and $\sigma^2_{\text{individual}}$. For all models, Markov Chain Monte Carlo parameters were set as follows: number of independent chains = three; number of iterations = 34,000; burning = 4000; thinning = three. We checked convergence through trace plot and autocorrelation along chains and results from the posterior distribution are reported as the half sample mode (HSM, Bickel and Frühwirth 2006) with 95% and 50% highest density intervals (HDI_{95}, Kruschke 2010). In Bayesian statistics, the HSM is a commonly used estimator of the central tendency of posterior probability distribution robust to outliers, whereas the HDI_{95} defines the interval that includes the parameter with 95% probability. That is because the posterior probability distribution represents the actual parameter value, given the data, and the HDI_{95} is constructed so that it contains 95% of this distribution. Parameter values in the center of the HDI tend to have higher credibility than parameter values at the limits. Therefore, when the HDIs of two groups do not overlap, there is a credible evidence for different group means. By contrast, to the extent the two groups' HDIs overlap there is evidence of no credible difference between the means. All analyses were done in R 3.6.1 (R Core Team 2019) using the packages R2jags (Su and Yajima 2015), modest (Poncet 2012), and HDInterval (Meredith and Kruschke 2018).

Results

In all trials males crossed into the side of the enclosure with the mirror (Nmirror range: 1–24), and in 75 cases (62.5%) they actually attempted to bite the mirror image. Overall, there was substantial variation by lizards in the time spent in the half of the arena with the mirror (618 ± 25 s, range: 33–1192, Table S1 for raw data). In the neutral treatment, males spent less time in front of the mirror (Time) and bit the mirror fewer times (Bites) than they did in the home treatment. Conversely, they increased the number of times they went into the arena half with the mirror (Nmirror) (Table 1, Fig. 1), with some differences depending on morphs, and the order of treatments (Fig. 1). In contrast, the number of tongue flicks did not vary according to treatment, morphs or order of stimulation (Table 1, Fig. 1). In all cases,

Table 1 Posterior distributions of the four response variables as estimated by LMMs. HSM and HDI_{95} estimates are shown

Covariates	Behavioural response			
	Time	Nmirror	Bites	TF
SVL	1.0 (-43, 44)	-0.8 (-1.7, 0)	-0.1 (-0.4, 0.2)	3.5 (-8.6, 15.5)
Day	22 (-22, 69)	-0.2 (-1.1, 0.7)	-0.1 (-0.4, 0.2)	2.9 (-9.6, 15.4)
Temperature	-22 (-61, 18)	-0.1 (-0.8, 0.7)	0.1 (-0.1, 0.4)	-0.3 (-10.6, 10.1)

Time time in seconds spent in the half arena containing the mirror; *Nmirror* the number of times the lizard entered the half of the arena containing the mirror, *Bites* the total number of bites against the mirror image (log-transformed, see text for details), and *TF* the number of tongue flicks in the half of the arena containing the mirror

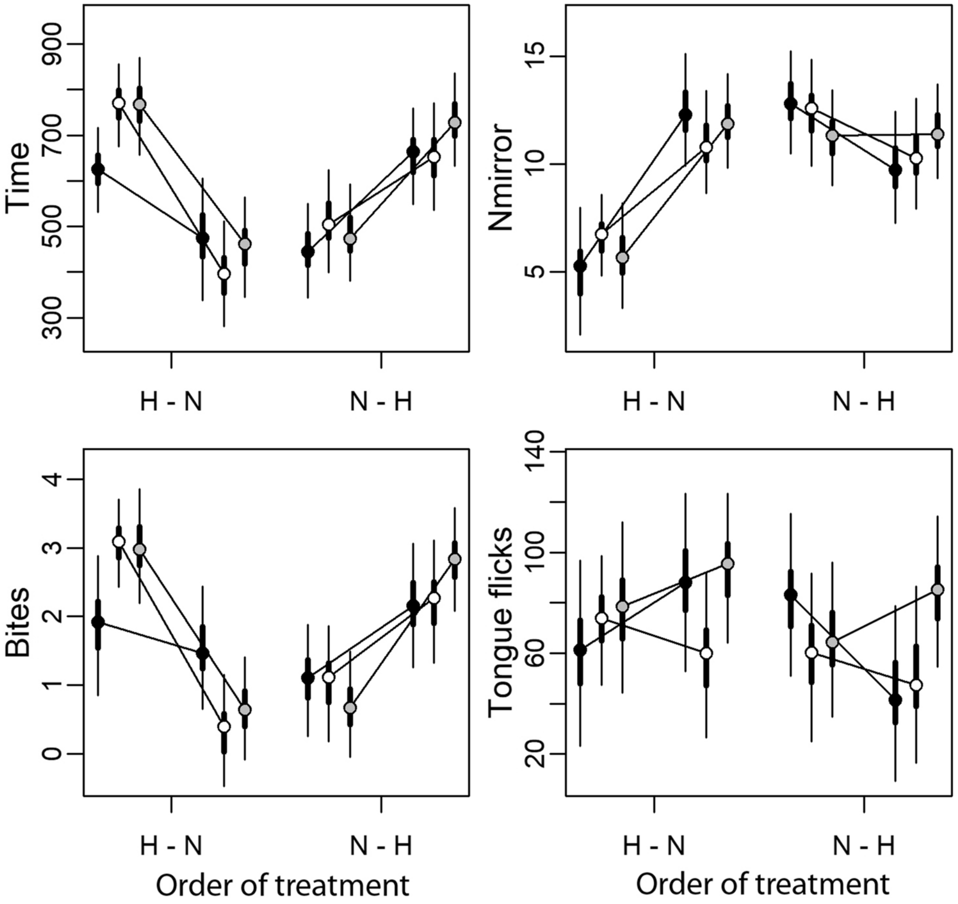

Fig. 1 Bayesian model predictions for the aggressive and exploratory responses of common wall lizards in home (H) vs neutral (N) arenas according to morph (*white*, *gray and black circles* are for white, yellow and red morphs, respectively) and experimental sequence. *Time* time in seconds spent in the half arena containing the mirror; *Nmirror* the number of times the lizard entered the half of the arena containing the mirror; *Bites* the total number of bites against the mirror image (log-transformed, see text for details); *TF* the number of tongue flicks in the half of the arena containing the mirror. *Circles* indicate HSM, and *thick and thin lines* represent HDI_{50} and HDI_{95}, respectively

no relevant effects were detected for body size (SVL), body temperature, or date of the trial (Table 1).

With respect to Time, the response was similar among morphs, and independent of the order in which we presented the treatments to males (Fig. 1). Indeed, males of all morphs spent more time in the half with the mirror in the home than in the neutral contest in both stimulation order (Fig. 1, Table 2), whereas each morph performed similarly within treatment independently of the orders of stimulation (Fig. 1, Table 3). Nevertheless, red males in the home contest spent less time in front of the mirror than both yellow and white males, but only in the home–neutral order of stimulation (Fig. 1, Table 4).

The order in which males were tested clearly predicted the number of times they entered the half of the arena hosting the mirror. Nmirror almost doubled in all three morphs when switching from home to neutral contests (Table 2), whereas no differences were found with the reverse sequence (Table 2). Furthermore, morphs performed similarly irrespective of both contests and order of stimulation (Table 3). Consequently, in the home arena trials males exhibited a higher response if done as the second trial, whereas in the

Table 2 Posterior distributions for the difference among the response variables estimated in home and neutral contests for each male morph according to the order (in brackets beside the treatment)

Variable	Home (first)–neutral (second)			Neutral (first)–home (second)		
	Red	White	Yellow	Red	White	Yellow
Time	**-153.5** (-295, -13)	**-373.8** (-516, -222)	**-310.6** (-465, -145)	**208.3** (76, 339)	**142.9** (-27, 310)	**251.1** (102, 404)
Nmirror	**7.5** (3.3, 11.6)	**4.3** (1.2, 7.4)	**6.2** (2.8, 9.6)	-2.9 (-6.5, 0.7)	-1.9 (-5.6, 1.8)	0.3(-2.9, 3.5)
Bites	-0.3 (-1.8, 1.1)	**-2.8** (-3.8, -1.7)	**-2.4** (-3.5, -1.2)	1.1 (-0.1, 2.4)	1.2 (-0.1, 2.4)	**2.1** (1.1, 3.2)
TF	27.7 (-23.4, 79.9)	-14.9 (-56.2, 27.1)	15.4 (-31.3, 60.6)	-38.4 (-85.1, 9)	-6.9 (-58, 42.6)	18.5 (-24.4, 60.9)

HSM and HDI_{95} estimates are shown. Bold values deviate from zero with $p \geq 0.95$. Variable names as in Table 1

Table 3 Posterior distributions for the difference among the response variables estimated in first and second trials (in brackets beside the treatment) in each experimental treatment for each male morph

Variable	Home (first)–home (second)			Neutral (first)–neutral (second)		
	Red	White	Yellow	Red	White	Yellow
Time	29.2 (-73, 132)	-113.5 (-235, 14)	-27.2 (-147, 99)	-19.3 (-159, 125)	52.7 (-105, 209)	58.3 (-108, 221)
Nmirror	**4.8** (1.5, 8.1)	**3.7** (0.8, 6.7)	**5.8** (2.9, 8.7)	-0.6 (-3.8, 2.7)	1.9 (-1.7, 5.4)	0.4 (-3.1, 3.9)
Bites	0.3 (-0.8, 1.5)	-0.9 (-1.9, 0.1)	-0.2 (-1.2, 0.8)	-0.9 (-2, 0.2)	0.7 (-0.5, 1.9)	0.4 (-0.8, 1.6)
TF	-16 (-57.3, 26.1)	-21.7 (-61, 17.4)	6.4 (-33.6, 46)	5.4 (-37, 48.9)	24.3 (-23.8, 72.2)	-35 (-82.6, 12.6)

HSM and HDI$_{95}$ estimates are shown. Bold values deviate from zero with $p \geq 0.95$. Variable names as in Table 1

Table 4 Posterior distributions for the difference among morphs for the response variables estimated in each trial within experimental treatment

	Red–white	Red–yellow	White–yellow	Red–white	Red–yellow	White–yellow
	Home (first)			Neutral (second)		
Time	**-140.9** (-248, -35)	**-136.8** (-251, -24)	3.8 (-119, 130)	78.3 (-78, 228)	19.3 (-126, 159)	-59.5 (-203, 87)
Nmirror	-1.6 (-4.9, 1.7)	-0.7 (-4.2, 2.9)	0.9 (-2, 3.8)	1.6 (-1.9, 5)	0.6 (-2.7, 3.8)	-1.0 (-4, 2.1)
Bites	**-1.2** (-2.3, -0.1)	**-1.2** (-2.4, 0.1)	0 (-1, 1)	**1.2** (0.1, 2.4)	0.9 (-0.2, 2)	-0.3 (-1.4, 0.7)
TF	-12.8 (-54.8, 29.2)	-18 (-64.9, 27.9)	-5.1 (-46.1, 35.4)	30 (-15.3, 75)	-5.4 (-48.9, 37)	-35.4 (-76.6, 5.9)
	Neutral (first)			Home (second)		
Time	-64.4 (-201, 72)	-37.6 (-174, 97)	26.3 (-116, 170)	2.1 (-141, 140)	-80.7 (-211, 48)	-82.1 (-226, 62)
Nmirror	0.5 (-2.7, 3.8)	1.6 (-1.5, 4.7)	1.1 (-2, 4.1)	-0.5 (-4, 2.9)	-1.7 (-4.9, 1.6)	-1.1 (-4.2, 2)
Bites	0.0 (-1.1, 1.1)	0.4 (-0.7, 1.4)	0.3 (-0.7, 1.4)	0.0 (-1.2, 1.2)	-0.7 (-1.8, 0.5)	-0.6 (-1.7, 0.4)
TF	24.2 (-19.3, 67.3)	16.9 (-25.7, 58.3)	-7.4 (-49.8, 34.5)	-7.3 (-53.9, 39.2)	-40.1 (-83.4, 2.7)	-32.8 (-76.2, 9.5)

The order of the treatment is shown in brackets. HSM and HDI$_{95}$ estimates are shown. Bold values deviate from zero with $p \geq 0.95$. Variable names as in Table 1

neutral contest no difference occurred between tests performed as first and second trials (Table 4, Fig. 1).

The number of bites to the mirror image decreased when switching from the home to the neutral contest irrespective of the order of presentation, and this effect was more/mostly evident in the yellow and white morphs (Table 2, Fig. 1). Indeed, red males assigned to the home–neutral treatment did not perform differently between first and second trials (Table 3), and performed fewer bites than both yellow and white males in the home contest, but more bites than white males in the neutral one (Table 4, Fig. 1).

Contrary to the aggressive-related variables, the posterior distributions of the number of tongue flicks largely overlapped among males, independently of the experimental contests (Table 2), the order in which the treatments were done (Table 3), and male morph (Table 4).

Finally, we found a relevant effect of the $\sigma^2_{\text{individual}}$ after controlling for the fixed effects, in all the response variables, suggesting the occurrence of an individual variability in both aggressive and exploratory behavior independent of morph and experimental treatment (Fig. 2). Such inter-individual variation accounted for at least 35% of the unexplained residual variance (HSM and HDI$_{50}$, Time: 40%, 1–47%, Nmirror: 35%, 4–48%; Bites: 38%, 6–51%; TF: 38%, 6–52%).

Discussion

The main aim of our study was to test if lizards escalate in a combat when their subjective RV is higher, after controlling for asymmetries in RHP and objective RV between the two opponents. By measuring the aggressive behavior in two opposite contexts, we clearly showed that lizards displayed a more intense aggression, and for a much longer time, when fighting for the territory of which they had a prior knowledge rather than for the territory not previously explored. Indeed, when switching from the neutral to the home arena, the interactions with the mirror image become longer and they involved a larger number of bites. A similar change in the exploratory behavior (i.e., the frequency of tongue flicks) did not occur, indicating that the shift observed for the aggressive behavior did not result from the experimental setting alone. Given that (i) the mirror removes any asymmetry in RHP between opponents, and (ii) the enclosures were identical (i.e., had the same objective RV), the only difference between the home and neutral trials was the subject's prior knowledge of the arena. So, we can conclude that the observed change in male aggressive behavior arose from a different subjective perception

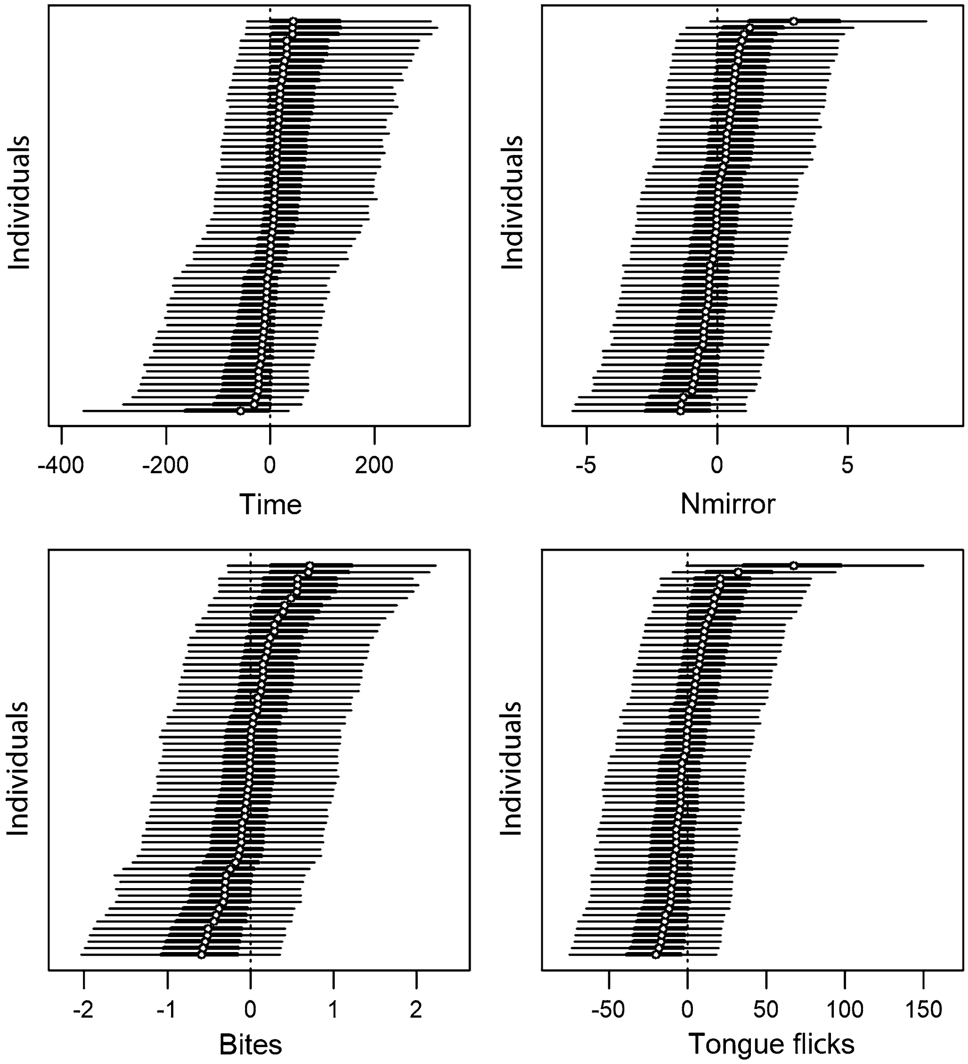

Fig. 2 Bayesian model predictions for the among-individual variation in aggressive and exploratory responses of common wall lizards. Variables as in Fig. 1. Each bar indicates each individual. *Circles* indicate HSM, and *thick and thin lines* represent HDI_{50} and HDI_{95}, respectively

that individuals have about the value of the contested resource. In other words, our data support the occurrence of a direct, positive correlation between motivation and aggression in this species. Motivation was already known to affect the contest outcome in common wall lizards (Sacchi et al. 2009), as well as in other lizard species (e.g., Stuart-Fox and Johnston 2005; Aragón et al. 2006; Carazo et al. 2008; Quintana and Gladino 2017). However, no previous studies have detected a direct correlation between subjective RV and aggression and its potential effects on the resolution of conflicts for indivisible resources. Indeed, there is general agreement that motivation in lizards can help males to overcome inferior RHP in conflicts due to residence advantage (Sacchi et al. 2009), winner–loser effect (Hsu et al. 2006) or dear–enemy effect (Carazo et al. 2008; Quintana and Gladino 2017). Unfortunately, the mechanisms which lead an individual to win over a superior opponent (in terms of RHP) have not been deeply examined yet, and the interaction between subjective RV and aggression could help us understand them.

Aggressiveness is a trait that affects the chances of winning a contest, but in a different way compared to RHP. The readiness of an individual to escalate (or to dare to escalate) during an encounter depends on an individuals' personality rather than on an individual's properties, and it is measured when the conflict is otherwise symmetrical (Barlow et al. 1986). Aggressiveness may have an even greater effect on individual fitness than RHP or RV, because willingness to escalate during an aggressive interaction may be more important in securing a territory than the ability of winning (Stamps and Krishnan 1994, 1998; Hurd 2006). In most animals, and even in common wall lizards, encounters strongly escalate when males are of similar size (Sacchi et al. 2009); in this situation, the more aggressive individual of the two opponents will increase its own possibility of winning the contest. Assuming a direct relationship between aggressive behavior and aggressiveness, the correlation between motivation and aggressiveness is likely to be the cause for the residency advantage, which was previously observed in common wall lizards. Indeed, smaller resident males are able to win when fighting with a larger intruder three times more frequently than smaller intruders fighting with a larger resident (Sacchi et al. 2009).

A second relevant result from this study was the high inter-individual variation in all variables related either to aggressive or exploratory behavior, as more than a third of the unexplained variance was associated with an individual's identity. In particular, this result suggests that aggressive behaviour varies among individual regardless of contest, supporting the idea that the inclination to attack a rival is related to aggressiveness, and ultimately the personality of each individual (Barlow et al. 1986). This result has important consequences on the general framework of assessment strategies and combat rules, as asymmetries in aggressiveness can arise independently of the opponents' motivation, as a result of differences in their personalities, and not because of a different subjective RV. On the other hand, higher motivation cannot always overcome inferior RHP if associated with a timid personality, or, at the exact opposite, an individual with lower motivation but a bold personality can still allow an individual to win if facing a shy opponent. In nearly 10% of asymmetric resident-intruder encounters of common wall lizard males, smaller intruders were able to win the contest against larger residents, when the differences in body size between contestants did not exceed 3.3% of the resident SVL (Sacchi et al. 2009). This outcome is not easily explicable only in connection to RHP and subjective RV asymmetries but becomes clearer if individuality of aggressiveness is accounted for. Indeed, the most plausible explanation is that successful intruders were individuals with a very aggressive personality, which were able to compensate for the greater motivation of the (larger) resident, given that the RHP asymmetries were not decisive for the outcome of the fight.

Game theory models suggest that aggressiveness may be more important than the ability to win fights in some species (Barlow et al. 1986; Maynard-Smith and Harper 1988), but simple strategic models of escalation have been criticized for attempts to empirically separate aggressiveness and variation in subjective RV because variation in aggressiveness may actually reflect long-term variation in subjective RV (Hurd 2006). This occurs because strategic models use the differences in payoff to predict the shift from threat display to fighting behavior, whereas aggressiveness does not affect the payoff (Hurd 2006). Consequently, the only way aggressiveness may affect the decision of escalating during a contest is when threatening and fighting have exactly the same payoff (Hurd 2006). In this paper, we were able to separate aggressiveness from subjective RV by modelling the first as a random effect and the second as a fixed effect in a linear mixed model. So, we found that if the subjective RV increases, the same occurs for the aggressive behavior and focal individuals escalate to fight as predicted by game theoretical models (Enquist et al. 1985). Simultaneously, we found that individuals significantly varied among one another for the basal level of the intensity of the aggressive display independently of the subjective RV, since the random effect actually compares the means of the response in the two contests among individuals. In other words, we separated the amount of the variability in the aggressive behavior due to the subjective RV from that due to the individuals (i.e., the aggressiveness). Nevertheless, measuring the aggressive response toward a mirror image does not allow us to measure a payoff because it is not possible to assess who is the winner, or, perhaps, the only possible outcome for the contestants is losing. This does not allow a reliable measure of benefits and costs for the contestants, and therefore the ability to model alternative strategies in terms of payoffs as in game theoretical models. Consequently, a measure of aggressive behavior using mirrors should not be able to disentangle the effects of either of RHP, RV, or aggressiveness from the others.

We did not find any evidence that throat and ventral coloration was related to male aggressive behavior despite the slight deviation of red males with respect to both white and yellow males for the time spent in front of the mirror and the number of bites to the mirror image. However, this difference appeared only in the group of males in which the sequence of stimulation was home–neutral, whereas no deviation among morphs occurred in the group stimulated with the neutral–home sequence. Thus, there was not enough difference to support the occurrence of a morph specific variation in the aggressiveness. Given the wide range of inter-individual variation in aggressiveness, it is rather more likely that the observed differences of the red morph might have been caused by a prevalence of shy individuals rather than any variability in aggressiveness on the basis of color morphs per se. Previous studies support a lack of correlation between morphs and aggressiveness. Sacchi et al. (2009) did not find any relationship between contest outcome and color morph in asymmetric resident–intruder encounters but did find a strong effect of asymmetries in RHP and motivation. Abalos et al. (2016) found that the ventral black patches emerged as a good predictor of contest outcome independently of morphs in pairwise contests in neutral arenas, even if red males lost fights against heteromorphic males more often than yellow or white males. However, this effect could be due to a correlation with the size of black patches. The plasma concentration of testosterone also did not differ among morphs (Sacchi et al. 2017), but only on the basis of the season. As a whole, these data do not support the hypothesis that aggressiveness varies among morphs.

There is increasing evidence that individuals do not use a unique assessment strategy during dyadic encounters, as has long been thought, but instead that assessment strategies can vary among individuals within populations and also within individuals during a conflict (Chapin et al. 2019). This is because the two opponents may not have the same source of information (Briffa and Elwood 2004; Arnott and Elwood 2009), may have different prior experience or knowledge (Briffa and Lane 2017; Camerlink et al. 2016), or may be differently influenced by external factors such as the distribution of resources, population demography, quality of information, ontogeny, and the expectation of future reproductive success (Chapin et al. 2019). The results of the present study show that assessment strategies for the resolution of animal contests in the common wall lizard can be very complex and combine characteristics at the species level, such as signals conveying information about RHP, with traits intrinsic to each individual, such as motivation and personality. More generally, we can say that our data show that one of three components involved in the resolution of dyadic conflict, the aggressiveness, varies from individual to individual, being a trait of individual personality. Interconnections between aggressiveness, motivation and personality have never been considered in the assessment strategies of dyadic encounters, probably because they are harder to model or because they act on a more hidden level than RHP or RV, but should attract much more attention as they represent the main source of variability in the process that leads an individual to choose to fight or retreat in a contest for an indivisible resource.

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CHAPTER 4

Morph-specific seasonal variation of aggressive behaviour in a polymorphic lizard species

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Morph-specific seasonal variation of aggressive behaviour in a polymorphic lizard species

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ABSTRACT

The persistence of colour polymorphism (CP) within a given population is generally associated with the coexistence of alternative reproductive strategies, each one involving specific trade-offs among behavioural, morphological, physiological, and other life histories. Common wall lizard (*Podarcis muralis*), is a medium-sized diurnal lizard, showing CP in three main colours (yellow, white, and red) on throat and belly, and a morph-specific pattern for both immunocompetence and seasonal variation of T levels. Yellow males show low stamina with high plasma T levels at the beginning of the season, while white males show high stamina with a higher plasma T levels at the end of the season. We hypothesised the presence of two strategies: a risky one, characterised by high aggressiveness played by yellow-morph, and a conservative one by white morph with low aggressiveness. Thus, we tested the aggressive response to conspecifics of yellow and white morphs using a mirror inserted into their cage, mimicking an intrusion of a stranger in their territories, throughout the breeding season (from April to July, 117 trials). We considered three types of aggressive response, with different levels of aggressiveness: (i) bite against the image reflected in the mirror, (ii) seconds spent by the individuals into the half mirrored cage, and (iii) number of times the lizard entered the half mirrored cage. We also considered the number of tongue flicking as explorative behaviour variable. All lizards were tested after a period of acclimatisation to the captivity conditions. Results demonstrate that yellow males showed a higher aggressive response in the early season and a decrease aggressive response towards the end, whereas white males showed an opposite pattern.

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Keywords Colour polymorphism, Aggressive behaviour, *Podarcis muralis*, Seasonal variation, Alternative strategies, Mirror test

INTRODUCTION

Species exhibiting colour polymorphism (CP) are excellent model systems to understand evolutionary processes. Following *Huxley's (1955)* definition CP is 'the presence of two or more genetically distinct forms which co-occur in both time and space, with the rarest form too common to be solely attributed to recurrent mutation'. This is a widespread

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phenomenon with extensive documentation in taxa through the Animal Kingdom, in both invertebrates and vertebrates (*Mckinnon & Pierotti, 2010*; *Wellenreuther, Svensson & Hansson, 2014*). CP can be maintained by natural and sexual selection, it is often genetically correlated, and the process which generates and maintains it can affect speciation and/or extinction rates, either positively or negatively (*Gray & Mckinnon, 2006*; *Hugall & Stuart-fox, 2012*). CP provides opportunities to explore how sexual selection works and how evolution maintains the coexistence of different phenotypes, since morphs represent an easily identifiable genotype-phenotype correspondence (*Mckinnon & Pierotti, 2010*). Within a given population, CP is generally associated with the coexistence of alternative reproductive strategies, which are modulated by complex interactions among environmental pressures (e.g. social interactions and individual density), each one involving specific trade-offs among behaviour, physiological, and other life-history traits (*Roulin & Bize, 2007*). Therefore, a colour morph represents an alternative, locally adapted optimum, that is an optimal combination of the traits involved in the trade-offs, which equally optimise the fitness (*Sinervo & Lively, 1996*; *Svensson, Sinervo & Comendant, 2001*; *Roulin & Bize, 2007*; *Sacchi et al., 2007a*; *Andrade et al., 2019*).

Trade-offs are usually thought in terms of resource allocation, where the allocation of limited resources to one trait has negative consequences for the other traits requiring the same resource (*Zera & Harshman, 2001*). Trade-offs involving the immune system are particularly interesting since the ability to deal with parasites and diseases has severe consequences on the fitness any individual may achieve, but also entails substantial costs for them (*Lochmiller & Deerenberg, 2000*; *Demas, 2004*; *Cox, Peaden & Cox, 2015*). A trade-off involving immunity is the one stated by the Immunocompetence Handicap Hypothesis (ICHH, *Folstad & Karter, 1992*), that assumes (and predicts) an inverse relationship between plasma testosterone (T) level and immune function. In lizards, the focus of our study, testosterone increases territoriality, home-range size and its quality (*Marler & Moore, 1988*; *DeNardo & Sinervo, 1994*; *Fox, 1983*), also affecting body shape and fertility in a variety of vertebrates (*Cox et al., 2005*; *Ketterson & Nolan, 1992*; *Oliveira, 2004*; *Hau, 2007*). Variation in plasma T level is related to aggressive behaviour in many species of vertebrates (*Rose, Holaday & Bernstein, 1971*; *Rada, Kellner & Winslow, 1976*; *Greenberg & Crews, 1983*; *Harding, 1983*). Aggressive behaviour leads to higher territorialism, which results in more females and more resources. Thus, high T levels would ultimately favour reproductive success (*Negro et al., 2010*; *Mokkonen et al., 2012*). However, maintaining high T levels has costs, non-only through the increased predatory risk associated to the T-driven behaviour, but also in terms of immune functions decrease, which favours parasite infections, and eventually degrades survival (*Olsson et al., 2000*; *Klukowski & Nelson, 2001*; *Cox & John-Adler, 2007*). Evidence in favour of ICHH has been found over different vertebrate taxa (*Roberts, Buchanan & Evans, 2004*; *Mills et al., 2008*; *Foo et al., 2016*).

Among vertebrates, reptiles represent a good model to investigate the evolution and maintenance of CP, particularly lizards (*Gray & Mckinnon, 2006*). In this clade, many species maintain marked CP at the population level and some underlying mechanisms have been already hypothesised and tested (*Sinervo & Lively, 1996*; *Thompson & Moore,* 1991; Thompson, Moore & Moore, 1993; Hews et al., 1997; Zamudio & Sinervo, 2000; Sinervo & Zamudio, 2001; Sacchi et al., 2007a; Huyghe et al., 2009; Runemark, Gabirot & Svensson, 2011). Notably, in males, the different breeding strategies often involve the modulation of aggressiveness against contending conspecifics according to the colour morph per se or to the morph combination of the focal and contending male (Sinervo & Lively, 1996; Hover, 1985; Thompson & Moore, 1991; Thompson, Moore & Moore, 1993). In many cases, the aggression level displayed and the contest outcome may be predicted by the colour morphs, irrespective of other asymmetries in size, residency, or prior experience (Sinervo & Lively, 1996; Hover, 1985; Thompson & Moore, 1991; Thompson, Moore & Moore, 1993). For instance, in the ornate tree lizard (*Urosaurus ornatus*), green males are more likely to dominate orange ones despite their smaller size (Hover, 1985); in the common blotched lizard (*Uta stansburiana*), orange males are highly aggressive over all other colour morphs (Sinervo & Lively, 1996), and in the painted dragon (*Ctenophorus pictus*), red males have a higher probability of winning the contest against yellow ones (Healey, Uller & Olsson, 2007). Further, in the above species, the more aggressive morph also showed higher absolute T levels (Knapp & Moore, 1997; Sinervo et al., 2000; Olsson et al., 2007). However, in other species of lizards, the link between CP and aggressiveness is less straightforward, and colouration appears to influence only the fight outcome between unfamiliar opponents (Stuart-Fox & Johnston, 2005; Sacchi et al., 2009).

On the immune side, the suppressive effect of testosterone is well-documented in many lizard species. An artificial increase in T levels decreases cell-mediated immunity (Olsson et al., 2000; Belliure, Smith & Sorci, 2004; Oppliger et al., 2004) and has been related to an increase of ectoparasites load and to hematological parameters variation (Puerta et al., 1996; Salvador et al., 1996, 1997; Veiga et al., 1998; Klukowski & Nelson, 2001; Pollock, Vredevoe & Taylor, 2012). In the light of these findings and since the alternative aggressive strategies constitute different fitness optima (Sinervo & Lively, 1996; Sinervo et al., 2000), we can hypothesise that the the trade-off stated by ICHH could play a role on the maintenance of the CP, where each morph is associated with a different behaviour strategy which consequently entails a connected immune response (Sacchi et al., 2009, 2017b).

The Common wall lizard (*Podarcis muralis*) is a medium-sized diurnal lizard (50–70 mm adult snout-vent length, SVL) that shows three main colour morph in both sexes, white, yellow, and red, on throat and belly (Sacchi et al., 2007b). The colourations develop from the second year of life (Cheylan, 1988) and the role of CP in social communication was widely studied in the last decades (Sacchi et al., 2009, 2015, 2017a, 2017b; Scali et al., 2013; Pellitteri-Rosa et al., 2014; Abalos et al., 2016; Pérez i de Lanuza, Carretero & Font, 2017; Mangiacotti et al., 2019a, 2019b). A morph-specific pattern in the immune response has been demonstrated (Sacchi et al., 2007a) and the same occured in plasma T levels throughout the breeding season (Sacchi et al., 2017b). These results show that yellow males are immunosuppressed compared to the other morphs, and bear higher plasma T levels at the beginning of the reproductive season (April), and lower at the end (July). These data suggest that the yellow males play a different strategy compared to the other colour morphs, that is yellow males invest more energy in aggressive interactions and intrasexual-competition at the beginning of the breeding season, at the expense of better stamina (as stated by ICHH); consequently, they are expected not to be able to maintain the needed aggressive level also in the late season (*Sacchi et al., 2017b*). However, a previous study found no morphs difference in aggressiveness (*Sacchi et al., 2009*), although there was a non-significant trend for red to lose when paired against yellow or white males (*Abalos et al., 2016*). Given these mixed results, and since the time of the breeding season was not considered, we conducted a study to examine if aggression varied in a morph-specific manner across the season. This question is motivated by documented morph-specific differences plasma T level and immune function, across the breeding season. In this study, we measured the aggressive response of male Common wall lizard morphs throughout the breeding season, to test the hypothesis of a morph-specific strategy, explicitly accounting for the time-dimension where the strategy is expected to be played.

MATERIALS AND METHODS

Lizards collection and housing

During spring and early summer 2018, we collected 117 adult males (69 white morph and 48 yellow morph, SVL > 50 mm) (*Sacchi et al., 2007b*) of Common wall lizard (*Podarcis muralis*) by noose in Pavia (Northern Italy, Lombardy). In order to track the whole reproductive season, captures were conducted every week from April to July, trying to balance colour morphs within each session, and at least two individuals each morph and session were collected. Since white and red males show the same T level seasonal pattern (*Sacchi et al., 2017b*), and red males occur at low frequency in Pavia populations (*Sacchi et al., 2007b*), we focused only on white and yellow morphs.

Within 2 hours of capture, the lizards were transferred to the University of Pavia, at the Department of Earth and Environmental Sciences, and housed in individuals Plexiglas cages (20 × 30 × 20 cm) with the four walls covered with white papersheets (to avoid visual disturbance during behavioural tests, see below). Each cage was provided with shelter, water ad libitum and lizards were fed with one mealworm (*Tenebrio molitor*) per day. Each lizard was measured (to the nearest 0.1 mm using a calliper) for snout-vent length (SVL) after the trial to reduce handling stress; the body mass (accuracy ± 0.1 g) was recorded at the capture time and after the trial, to assess that the housing protocol had no effect ($P = 0.06$). Mean SVL was 63.4 ± 3.0 mm (range 57.5–72.0 mm) for the yellow morph and 63.0 ± 3.5 mm (range 55.8–71.0 mm) for the white morph. There was no difference in size between morphs (two samples t-test, $t = 0.69$, df = 115, $P = 0.49$). The housing room was maintained between 15 and 32 °C, simulating the temperature range observed in Pavia in late spring and early summer (*Karger et al., 2017*), and natural daylight was guaranteed. Trials started after an acclimation period of at least 7 days, to allow individuals to consider the cage as their own territory (*Mangiacotti et al., 2019b*). All lizards were released, healthy, at their captured sites, within a maximum of 2 weeks from their capture.

Experimental setting

To measure the aggressive response, we used a mirror test to mimick an intrusion of a stranger in the individual's territory (the cage). This method allows removing the size and motivation effects by showing to the tested individual an image with the same behaviour, size, and motivation (*Sacchi et al., 2009*; *Scali et al., 2019*, *2020*). After acclimation, lizards were tested in their own cage after removing the water bowl. To avoid visual disturbance during the experiments, the four sides of the cage were externally covered with white paper. The experimental protocol consisted in: (i) heating the lizard in its cage for 5 min with a 75 W halogen infrared lamp positioned 40 cm above the cage; (ii) inserting the mirror, covered by a plastic septum, in the cage wall opposite to the shelter; (iii) after 3 more minutes, removing the septum and recording lizard behaviour, using a webcam (Microsoft LifeCam HD 3000) positioned above the cage and connected to a laptop. Recording duration was set to 15 min and started at the first exploratory movements (i.e. tongue flicking, head movements towards the mirror, etc.). Videos were managed by Free2X software v1.0.0.1 (freely available at http://www.free2x.com/webcam-recorder/), setting quality to 800 × 600 pixels and 15 fps (*frames per second*). The trials were run between 10 a.m. and 2 p.m., and the order of morphs was randomised to remove any potential effect of day-time. At the end of each trial we measured the body temperature of lizards with a handheld infra-red thermometer (Lafayette TRP-39, Lafayette Instrument Co., Lafayette, IN, USA; sensitivity: 0.1 °C; precision: ±2%)

Response variables

We processed all videos using BORIS open-source software (Behavioral Observation Research Interactive Software, available at www.boris.unito.it, *Friard & Gamba, 2016*), which allowed us to extract from each video an ethogram consisting in four response variables: (i) the total number of bites against the reflected image (Bites); (ii) the time spent in the half mirrored cage (Time, in s); (iii) the number of times each individual entered the half mirrored cages (Nmirror); (iv) the ratio of the number of tongue flicks to Time (RTF). While the first three variables were considered proxies for different levels of aggressive behaviour, the fourth one evaluated the basal explorative behaviour of each individual when facing a potential contestant (Sacchi et al., 2020, unpublished data). In particular, we considered Bites as the maximum level of aggressiveness, such as direct aggression to the 'rival'; Nmirror as the interest in facing the 'rival', and Time as the interest for the 'rival', since the time spent in the half mirrored cage would have been the same as the one spent in the other half if the mirrored image did not elicit the interest of the focal male: the longer the time, the larger is the interest (Sacchi et al., 2020 unpublished data). All variables were weakly correlated with each other (Spearman correlation coefficient: $|r_{Spearman}|_{max} = 0.31$).

Statistical analysis

Time, Nmirror, and RTF assumed a normal distribution (One-sample Kolmogorov-Smirnov test, all *P* values larger than 0.05), while Bites showed a Poisson-like distribution with overdispersion (sd/mean = 34), and zero inflation. Thus, we ran Zero-Inflated

Table 1 **Descriptive statistics of the results of the behavioural experiments.**

	White	Yellow	Total
Bites*	13 (11–64) (0–116)	5 (1–35) (0–92)	8 (3–50) (0–116)
NMirror	6 ± 0.4 (1–19)	6.3 ± 0.6 (0–20)	6.2 ± 0.35 (0–20)
Time	805 ± 33 (54–1,199)	759 ± 46 (0–1,174)	786 ± 27 (0–1,199)
Ratio of Tongue Flicking	0.09 ± 0.01 (0–0.28)	0.09 ± 0.01 (0–0.23)	0.09 ± 0.01 (0–0.28)

Note:
Mean (min–max) values of each response variable. Asterisk (*) is for Poisson distributed variables and the mode is indicated with the first and third quartiles. For all variables, the range is indicated (min–max) above.

Negative Binomial Regression (ZINB) for Bites and a Linear Model (LM) for Time, Nmirror, and RTF. Julian date (hereafter Day), morphs and their interaction entered the model as fixed effects, in order to assess whether the response variables varied over the season but with different patterns between morphs. We also added SVL and body temperature as a fixed effect to control for possible confounding effects due to age and individual activity. All analyses were done in R 3.6.2 (*R Core Team, 2019*) using the package glmmABDM (*Fournier et al., 2012*), and otherwise stated, data reported are means ± standard errors. The study was performed following the European and Italian laws on animal use in scientific research, and all the protocols have been authorised by the Italian Environmental Ministry (Aut. Prot. PNM0002154.03-02-2016, valid for the 3 years 2016–2018).

RESULTS

Lizards approached the mirror in 116 out of 117 trials (99.1%), and bit the mirrored image in 82% of cases. The time spent in the half mirrored cage ranged from 0 to 1,198 s, being on average 786 s, and lizards entered the half portion of the cage hosting the mirror on average 6.2 times each trial (range 0–20, Table1). Nmirror was on average 6 ± 0.4 for white males (range 1–19) and 6.3 ± 0.6 for yellow males (0–20); the mean for Time was 805 ± 33 s (range 54–1,199) for white males and 759 ± 46 s (0–1,174) for yellow males. RTF was identical in the two morphs (Table 1). Finally, the most frequent number of bites to the mirror (mode and interquartile distance) was 13 (11–64) and 5 (1–35) in white and yellow males respectively (Table 1).

The statistical analysis showed that Bites significantly varied depending on the Day (then, with the season), morphs, and their interaction (Table 2), suggesting that the aggressive behaviour changes during the season with a morph specific pattern. A significant increase in bites has been observed in white males (Day: $\beta = 0.37 \pm 0.16$; $P = 0.02$), whereas the opposite pattern has been observed in yellow males, although not in a significant statistical way (Day: $\beta = -0.29 \pm 0.16$; $P = 0.07$). Specifically, yellow males at the beginning of the season had higher aggression than white males, but as the season went on this difference disappeared until a switch of the aggressive behaviour in the two morphs did occur. Consequently, late in the season, white males were more aggressive

Table 2 Results of statistical analysis.

	Df	X^2	*P*
Bites			
Day	1	5.68	**0.02**
Morph	1	4.78	**0.03**
SVL	1	0.58	0.45
Temperature	1	1.38	0.24
Day:morph	1	9.31	**0.002**
Time			
Day	1	0.73	0.39
Morph	1	1.35	0.25
SVL	1	2.06	0.15
Temperature	1	2.13	0.14
Day:morph	1	0.30	0.59
Nmirror			
Day	1	0.07	0.80
Morph	1	0.33	0.56
SVL	1	0.07	0.79
Temperature	1	0.76	0.38
Day:morph	1	1.31	0.25
Ratio of tongue flicking			
Day	1	0.01	0.77
Morph	1	0.12	0.73
SVL	1	0.10	0.75
Temperature	1	1.90	0.17
Day:morph	1	0.08	0.78

Note:
Effects of the experiment date, morphs, SVL, final temperature, and interaction Day × morph on Bites, Time, Nmirror, and Ratio of Tongue Flicking in males of *Podarcis muralis*. Significant *P* values are reported in bold.

to the mirror than yellow males (Fig. 1). Finally, our results show no significant effect of temperature and size for Bites (Table 2).

The statistical analysis performed for Time, Nmirror and RTF did not show any statistically significant effect of both Day and colour morph (Table 2): individuals explored the space in the same way regardless of size, season, temperature, or morphs.

DISCUSSION

In this study we measured the variation of aggressive response during the breeding season in *P. muralis* males using a mirror, mimicking an intrusion of a stranger in the lizards' territory. Our experiment clearly showed that yellow males were more aggressive (i.e. bit their mirrored image) than white males early in the season, but this difference reversed as the season progressed. The lack of a significant difference in the explorative behaviour, that is RTF, between morphs along the season, supports the idea that seasonal variation in aggression was not due to the experimental setting. The results of this study agree with the hypothesis that aggressive behaviour is a morph specific trait, and

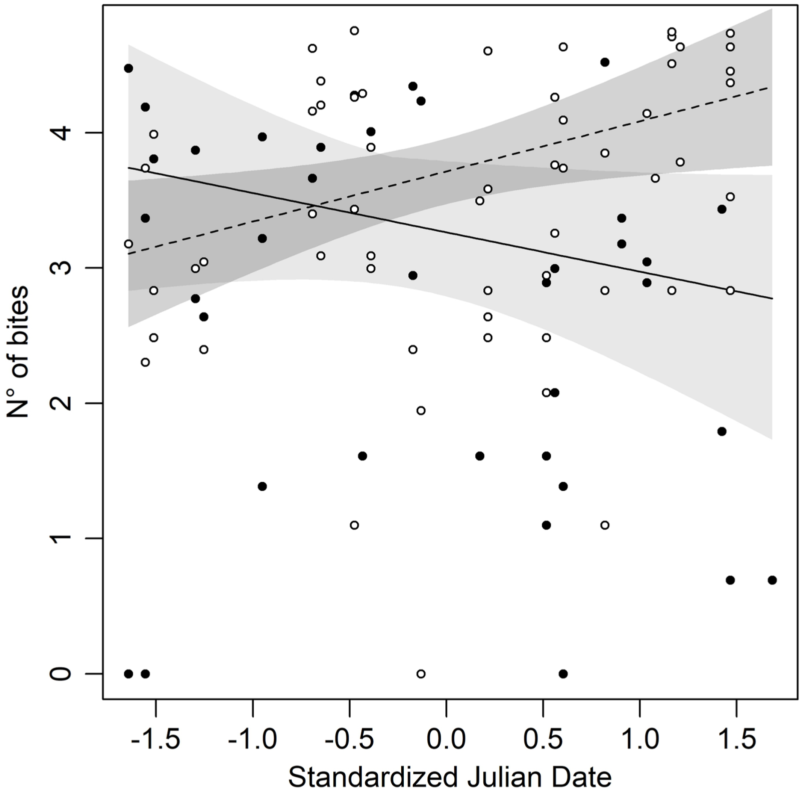

Figure 1 **Number of bites against the mirror throughout the season in both morphs of Common wall lizard.** Yellow morph: black dot = observed values, dashed line = predicted line, light grey area = 95% confidence interval; white morph: white dot = observed values, continuous line = predicted line, dark grey area = 95% confidence intervals. Full-size DOI: 10.7717/peerj.10268/fig-1

it is displayed with different patterns in the two morph according to the specific breeding strategies they adopt. However, only the highest expression of aggressive behaviour (i.e. Bites) showed this seasonal, morph specific trend (increases in whites and decreases in yellows). Indeed, we did not find any significant difference in both Nmirror and Time, which measured a lower-level aggressive expression. Nmirror and Time represent threatening attitudes at distance, which did not imply a direct physical interaction with the opponent, and consequently no risk of injuries. So, the costs of those displays directly affecting the ICHH trade-off are probably low, and, therefore, are not relevant in shaping the morphs' alternative strategies. By contrast, overt aggression, that is Bites, has potentially high costs in terms of both injuries and stress. Among all the variables, our result shows that only 'Bite' varied seasonally, and in a morph-specific manner, an this supports the hypothesis that they modulate the ICHH trade-off in two different ways (i.e. with two alternative strategies), depending on the costs they are willing to sustain during aggressive interactions with conspecific males.

Male Common wall lizards can recognise individuals belonging to their morph (*Pérez i de Lanuza et al., 2018*) and they show higher aggression towards individuals belonging to their same morph (*Scali et al., 2020*). They also show greater aggressiveness in a familiar context (i.e. their territory) with a high subjective resource value than in unfamiliar context

(*Sacchi et al., 2009*; Sacchi et al., 2020 unpublished data). Having said that, our experimental setting reflect the most stimulating context, regard with Resource-Holding-Potential and morph-specific aggressive pattern (*Sacchi et al., 2009*; *Scali et al., 2020*; Sacchi et al., 2020 unpublished data), which allows to consider the obtained results as the maximum possible aggressive response in a given period. These results agree with the morph-specific seasonal pattern in plasma T levels found in *Sacchi et al. (2017b)* and support our initial hypothesis which envisaged the existence of a more aggressive strategy (played by yellow males) as opposed to a more conservative one (played by white males). Increased aggressive behaviour in the early part of the season means more clashes among individuals, but at the cost of lower long-term survival due to higher predatory risk and a lower immune response (*Marler & Moore, 1988*; *Sacchi et al., 2009*) to the benefits of those who choose the 'conservative strategy'. Since aggressive behaviour has an inverse correlation with the immune response, as stated by ICHH, we can say that these alternative strategies come out by a trade-off between two contrasting needs: the investment in territorial aggression on one hand and a longer survival on the other.

Adopting a different strategy depending on morphs can help individuals to recognise the strategy of rivals and modulate their own. Many species of vertebrates show an aggressive morph-specific response: an example is given by cichlids fish *Metriaclima mbenjii* where males direct more aggression towards similarly coloured opponents (*Van Doorn, Dieckmann & Weissing, 2004*; *Seehausen & Schluter, 2004*; *Dijkstra et al., 2006*, *2007*, *2008*, *2010*; *Pauers et al., 2008*) and similar results happened in the polymorphic sparrow, *Zonotrichia albicollis* (*Horton, Hauber & Maney, 2012*). It is also recurrent in reptiles: in *Urosaurus ornatus* males the manipulation of colour triggers aggression against opponents (*Hover, 1985*); experiments performed with colour-manipulated models of *Ctenophorus decresii* showed a higher aggressive behaviour during homomorphic contexts (*Yewers, Pryke & Stuart-Fox, 2016*). Former studies concerning aggressive morph-specific response as a mechanism underlying polymorphism maintenance in Common wall lizard showed contrasting results. *Sacchi et al. (2009)* did not find any correlations between the aggressive strategy and individual's morph; instead, *Abalos et al. (2016)* found a lower fighting ability in red males, but this could be due to the size of black patches. Finally, *Scali et al. (2020)* demonstrated that *P. muralis* shows a greater aggressive response if the contender belongs to its own morph. The results of our experiments clearly show that aggression is modulated over time by morphs, and it is important to consider the time window when carrying out this type of behavioural experiment because it would provide different seasonal results based on the morph. For example, if you carry out behavioural experiments too close to the inversion point (Fig. 1) you will not notice aggressive response differences between morphs, resulting in a false negative.

High aggression leads to a greater chance of winning the encounters and consequently a greater chance of breeding. The existence of alternative strategies could be due to the opportunity of males to invest in different clutches. Previous studies on reproductive biology of lacertid lizards, and in particular of *P. muralis*, indicate that female lizards show two peaks of deposition in late April and late May (*Sacchi et al., 2012*; *Galeotti et al., 2013*).

Moreover, material for yolk production of the first clutch is mainly derived from fat reserves stored before hibernation, whereas subsequent clutches are influenced by the available resources in the current season (*Braña, Gonzalez & Barahona, 1992*). So, the seasonal aggressiveness modulation in male morphs may synchronise with female deposition timing, suggesting that yellow males may aim at the first clutch (based on female fat reserves), whereas white males at the following ones (depending on resource availability along the season).

CONCLUSIONS

Our study shows that seasonal pattern of variation in aggression in male Common wall lizards is morph specific and in accordance with the prediction of the trade-off promoted by the ICHH. According to it, white and yellow males of this species could adopt two alternative strategies involving different investment in aggression and immunity function leading to a riskier strategy (yellow males) and a more conservative one (white males). Yellow males tend to be more aggressive at the beginning of the season, while white males are able to maintain an overall higher aggressiveness along the season. The different phenology of the aggressive behaviour between male morphs may help to explain the persistence and coexistence of different morphs in a population.

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Alan Jioele Coladonato conceived and designed the experiments, analysed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

- Marco Mangiacotti conceived and designed the experiments, analysed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Stefano Scali conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Marco A.L. Zuffi conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Carlotta Pasquariello performed the experiments, prepared figures and/or tables, collecting samples, and approved the final draft.
- Cristian Matellini performed the experiments, prepared figures and/or tables, collecting samples, and approved the final draft.
- Simone Buratti performed the experiments, analysed the data, prepared figures and/or tables, collecting samples, and approved the final draft.
- Mara Battaiola performed the experiments, prepared figures and/or tables, collecting samples, and approved the final draft.
- Roberto Sacchi conceived and designed the experiments, analysed the data, authored or reviewed drafts of the paper, and approved the final draft.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

Italian Environmental Ministry (Aut. Prot. PNM0002154.03-02-2016) authorised this study.

Data Availability

The following information was supplied regarding data availability:

All data that we used for statistical analysis are available in the Supplemental Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.10268#supplemental-information.

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CHAPTER 5

Effect of testosterone manipulation in aggressive response and immunocompetence in a polymorphic lizard

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In submission

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ABSTRACT

The persistence of colour polymorphism (CP) within a given population is generally associated with the coexistence of alternative reproductive strategies, each one involving specific trade-offs among behavioural, morphological, physiological, and other life histories. Trade-offs involving immune system are of particular interest since the ability to deal with parasites and diseases has severe consequences on the fitness individuals may achieve, but also entails substantial costs for them. T level fosters a strong motivation to defend territories against rivals (i.e. increasing movements, activity, and aggressive display) and increases mating. At the same time, high T level can decrease immune functions, favours parasite infections, stimulates risky behaviours, and thus diminishes survival, according to trade-off stated by ImmunoCompetence Handicap Hypothesis (ICHH), and there is no way to maximize stamina and aggressive behaviours at once. The evolution and the maintenance of CP have been studied in many species of Reptiles, particularly lizards, that represent a good study model among vertebrates. Males of many species often modulate aggressiveness with a morph-specific pattern. Common wall lizard (*Podarcis muralis*), is a medium-sized diurnal lizard, showing CP in three main colours (yellow, white, and red) on throat and belly, and a morph-specific pattern for immunocompetence, seasonal variation of T levels and seasonal variation of aggressive response. Yellow males had a higher T levels-correlated aggressiveness, in respect to the white and red males in the first part of breeding season. T levels and aggressive response displayed a stronger subsequent decline towards the end of the season, and white males became more aggressive. Farther, yellow males show low stamina, while males of the other two morphs show high stamina. We hypothesised the presence of two alternative strategies, where yellow morph aims for sexual behavior traits, such as aggressiveness, while the white morphs aim to maximize the ability to deal with diseases and parasitism. To tested this hypothesis we experimentally manipulated plasma T levels of 40 males with a non-invasive method and measured aggressive response and immune response before and after the treatment. Results demonstrated that yellow males shown a stronger immunosuppressed in respect to white males, and this affects negatively the aggressive response. Yellow and white morphs seem like the opposites of the trade-off predict by ICHH.

INTRODUCTION

When in a population, individuals of the same-age and same-sex display two or more colour variants (morphs), genetically inherited, and for which the expression is neither sensitive, or only to a low extent, to the environment nor to body condition, we can talk of Colour Polymorphism (CP; (Ford, 1945; Huxley, 1955; Buckley, 1987). Studies of last decades provide that CP is maintained by several factors, such as disruptive selections, apostatic and sexual selection, genetic drift and gene flow, heterosis, sensory bias and predation that would benefit rare morphs (Sinervo & Zamudio, 2001; Galeotti et al., 2003; Roulin, 2004; Gray & Mckinnon, 2006; QvarnstrÖm, Vallin & Rudh, 2012). Thus, CPs are powerful model systems to study the evolutionary processes, since morphs are generally associated with alternative reproductive strategies, that come out from trade-offs among different organismal functions for limited internal resources (Roulin & Bize, 2007). When two or more life-history traits compete, it is interesting to understand how trade-offs optimize resource allocation (Zera & Harshman, 2001). A very interesting trade-off is the one that comes out among two important life history traits: hormones and immune function. ImmunoCompetence Handicap Hypothesis (ICHH) affirms that the primary androgenic hormone, testosterone, is in a negative correlation with ImmunoCompetence (IC; Folstad & Karter, 1992). Plasma Testosterone (T) level is involved in many traits that lead males to reproductive success (Fox, 1983; DeNardo & Sinervo, 1994; Negro et al., 2010; Mokkonen et al., 2012), such as body shape, behaviour (i.e., aggressive and territoriality), fertility and development (Ketterson & Nolan, 1992; Brantley, Marchaterre & Bass, 1993; Oliveira, 2004; Cox et al., 2005; Hau, 2007). On the other hand IC is important for individuals' survival, since wild animals live with chronic, life-long infection and parasites coevolving with their hosts indicating that this is not exception, but the norm (Ressel & Schall, 1989; Sinervo & Lively, 1996; Moret & Schmid-Hempel, 2000). As ICHH stated, an increase of T levels leads a decrease of IC in vertebrates with eventually a decrease in survival (Olsson et al., 2000; Klukowski & Nelson, 2001; Roberts, Buchanan & Evans, 2004; Cox & John-

Adler, 2007; Mills et al., 2008; Foo et al., 2016). Trade-offs given from ICHH can explain how CP has been maintained.

Among vertebrates reptiles, and particularly lizards, offer a good model to study the interaction between hormone and immune system (Salvador et al., 1996; Olsson et al., 2000; Cox & John-Adler, 2007) and the evolution and maintenance of CP (Sinervo & Lively, 1996; Smith & French, 2017). The effect of experimentally T levels manipulation is well documented in many lizard species: male individuals obtained a larger with more quality home range and are more successful in conflicts against opponents (Fox, 1983; Moore & Marler, 1987; DeNardo & Sinervo, 1994). However, increase plasma T levels lead a decreases cell-mediated immunity (Olsson et al., 2000; Oppliger et al., 2004).

Common wall lizard (*Podarcis muralis*) is a small diurnal lizard widespread in central and southern Europe (Sillero, 2004). This species exhibits three discrete colour morphs (yellow, white and red) genetically determined in both sexes (Sacchi et al., 2007b; Andrade et al., 2019). Studies of last decades found morph-specific patterns concerning some traits of life-histories. In particular, yellow males compared to the other morphs are immunosuppressed (Sacchi et al., 2007a), and bear high plasma T levels at the beginning of breeding season displaying a stronger subsequent decline towards the end (Sacchi et al., 2017b). These levels match with seasonal aggressive behaviour (Coladonato et al., 2020). In fact, plasma T levels did not differ among morphs, but varied seasonally and the aggressive response has shown the same pattern. Yellow males had a higher T levels-correlated aggressiveness, in respect to the white and red males in the first part of breeding season. T levels and aggressive response displayed a stronger subsequent decline towards the end of the season, and white males became more aggressive (Sacchi et al., 2017b; Coladonato et al., 2020). For this reason, we could hypothesize the presence of two distinct strategy: a risky strategy played by yellow males, where they invest in more aggressive behavior despite the immune function, and in a conservative strategy played by no-yellow males, where the fitness is achieved with greater survival. Thus, the maintenance of CP in this species could be modulated by a trade-off according to the ICHH paradigm.

T manipulation has already been successfully tested in this species in previous work (Oppliger et al., 2004; Baeckens & Van Damme, 2018), showing a correlation between increased testosterone and a decrease in fitness, in terms of decrease cell-mediated response and increased mean metabolic rate (Oppliger et al., 2004), but any work tested a morph-specific pattern of immune response after T manipulation, but always using a correlation way (Sacchi et al., 2007a) and an experimental approach are needed to assess any physiological correlation.

In the present study, we wanted to measure the presence of differences among morphs in the T-cell mediated immune response and aggressive behaviour of Common wall lizards, before and after experimental plasm T levels increase. To do this, we set up cell cultures from blood samples that were activated with phytohaemagglutinin (PHA) and incubated for three days, to test the immunocompetence, and using a mirror to mimic the intrusion into the territory by a stranger, to test aggressive response. Then, we used a non-invasive technique, already successfully used in this species, to increase plasma T levels in yellow and white morphs males and re-measured immunocompetence and aggressive behaviour at the end of treatment. We expect the immune response (already tried in this species, but without considering the colors) (Oppliger et al., 2004), and therefore the aggressive response, to be different depending on the treatment, as predicted by the ICHH. Furthermore, considering that the morphs are genetically set to have different testosterone levels throughout the season (Sacchi et al., 2017b; Andrade et al., 2019), we expect that the increase in testosterone affects the two responses differently depending on the morph.

MATERIAL & METHODS

Sampling and housing lizards

We captured by noosing 40 adult males of *Podarcis muralis* (20 white and 20 yellow, SVL 59-70mm) (Sacchi et al., 2007b), at the end of March 2019 (from the 20th to the 26th), around Pavia town (Northern Italy, Lombardy). We only focused on white and yellow morphs because white and red males show the same T level seasonal pattern and the same immune-function (Sacchi et al., 2007a, 2017b), but red males occur at low frequency in Pavia population (Sacchi et al., 2007b). Lizards were carried to Freshwater and Herpetology Laboratory of Department of Earth and Environmental Science (University of Pavia) after capture and housed in individual plastic boxes (20x30x20). These boxes contained a brick as shelter, a little bowl with water *ad libitum*, a UVB 5% lamp turned on 8 hours/day (Sylvania F30W Reptistar T8 UVB 5%), and heating pads as hot spot. Individuals were fed with mealworms (one/day), which were dusted with vitamin and calcium supplements two times for week, and natural daylight was provided. Before housing started, we measured snout-to-vent length (SVL) with a caliper (to the nearest 0.1) and weighed each male, and the weight was recorded also at the and the experiment to access the experimental protocol and housing had no effects (*P*>0,05). Experiments started after an acclimation period of at least 7 days, to allow individuals to consider the cage as their own territory (Mangiacotti et al., 2019). At the end of experiment, all lizards were released at the same capture site looked healthy.

Manipulation of testosterone

We used for this work a non-invasive technique already proposed for other lacertid species (Knapp & Moore, 1997; Belliure, Smith & Sorci, 2004; Oppliger et al., 2004) and successfully used in *P. muralis* (Baeckens et al., 2017) to increase plasma T levels. Thus, we gave a solution of commercial sesame oil and testosterone powder (4- androsten-17b-ol-3-one; Sigma #86500) to the males every two days for four weeks. The concentration of solution was 4 μg of testosterone powder/μL of sesame oil, and we applied 4 μL on the back of lizards in the early morning, before turning on UVB lamp and heating pad, when the individuals were not in activity, to minimize the stress. To assess that the T levels were increased by treatment, we measured the hormone concentration using a non-invasive steroid analysis based on faecal dropping, as described in Palme (2005; 2013). We collected faecal drops for each individual before the treatment started and at the end, freezing the samples as fresh as possible and stored them in individual labeled Eppendorf tubes 0,5 mL at -20°C. The enzyme radio immune assay to measure the T concentration was performed at Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine in Vienna.

Behavioural experiment

Each individual performed double behavioural experiments to measure the aggressive response: before and after the hormone treatment. We used a mirror inserted into the box to mimick intrusion of a stranger in their own territory, as described in Scali et al. (2019). We removed the water bowl during the trial. The experimental protocol provided for these phases: i) we heated lizard with a 75W halogen infrared lap placed 40cm above the cage, until the activity temperature is reached (38°C; Sannolo et al., 2014); ii) we placed a mirror in the opposite wall of shelter. At first, the mirror was covered by a plastic septum; iii) after three minutes, we removed the septum. Test lasted 15 minutes, starting at first lizard movement (i.e., tongue flicking, head movement, etc.), and we recorded all the activities with a webcam (Microsoft LifeCam HD 3000) fixed above the box and connected to a laptop. The videos were managed by Free2X software (open source, available at http://www.free2x.com/webcam-recorder/). Trials took place between 10 a.m. and 2 p.m., and the order of morphs was randomized to control for potential effects of day-time. After trial, we measured the body temperature with a handheld infra-red thermometer (Lafayette TRP-39, Lafayette Instrument Co., Lafayette, Indiana, USA; sensitivity: 0.1°C; precision: ± 2%).

Response variables

All videos were analysed with B.O.R.I.S (Friard & Gamba, 2016), and we extracted four behaviour variables: three aggressive responses with three different levels of aggression (Scali et al, 2020, Coladonato et al., 2020; Sacchi et al., 2021), and basal explorative behaviour. In details, the first three variables were:

- *Nmirror*: number of times that lizards entered in the half mirrored box. We considered that as the individual facing a 'rival';
- *Time*: seconds spent from individual in the half mirrored box as individual's actual interest to 'rival'. If the mirrored image did not elicit the interest of focal male, we supposed the time spent in the half mirrored cage would have been the same as the one spent in the other half: the longer the time, the larger is the interest;
- *Bites*: bites against the mirrored image, the highest level of aggression (i.e., a direct aggression to the "rival").

The last one was:

- *RTF*: the ratio of tongue flicking to *Time*, hence taking into account only those in half mirrored box. This variable evaluated the basal explorative behaviour of each individual when facing a potential contestant.

All variables were weakly correlated with each other (Spearman correlation coefficient: $\left|r_{Spearman}\right|_{max}$= 52).

Immuno-response test

In order to evaluate the immune response from yellow and white males, we carried out two *in vitro* activation of lymphocytes, one before the T manipulation treatment and one at the end. For each test, and for each individual, we collected 20µL of blood with heparinized capillary tubes from post-orbital sinus, and inoculated in 15ml of RPMI 1640 medium supplemented with 10% bovine serum. Then, we divided the cell suspension into two 7ml sub-cultures, one of which was inoculated with 1% PHA solution (PHA-P Sigma, 50mg in 10ml of phosphate-buffered saline) (Oppliger et al., 2004; Sacchi et al., 2014, 2017a). The last 1ml of solution was used to assess starting lymphocyte concentration using a Neubauer chamber. Each sub-culture was then distributed in two 1.5ml culture tubes, and incubated at 32°C for 3 days (Sacchi et al., 2014, 2017a). Afterward, cells were collected, re-suspended and newly counted. This second count involved only proliferating lymphocytes. Stimulating of T-cell after incubation was evaluated by determining the colony-forming units (cfu) per ml (PHA_cfu) to the corresponding control and an estimate of the total cells of each cfu (PHA_tot).

Statistical analysis

First at all, we assessed the increase of faecal T concentration following treatment using a linear mixed-effects model. Bites showed a Poisson-like distribution with overdispersion (sd/mean = 29), and zero inflation, while the other variables assumed a normal distribution (One-sample Kolmogorov- Smirnov test, all P values larger than 0.05). Thus, we ran a Zero-Inflate Negative Binomial Regression for Bites, and linear mixed-effects model via Satterthwaite's degrees of freedom for the others variable. 'Treatment' (i.e. before and after the T levels manipulation), morphs and their interaction was used as a fixed effect, to verify whether the responses were affected by T levels increase, SVL as a fixed effect to control for possible confounding effects due to age. Finally, the lizards' ID entered as random effect. We also tested the correlation among aggressiveness (Bites), immunity (PHA_cfu) and T with a Zero-Inflated Negative Binomial Regression. All analyses were done in R 3.6.2 (R Core Team, 2020) using packages lmerTest (Kuznetsova, Brockhoff & Christensen, 2017) and glmmADMB (Fournier et al., 2012).

RESULTS

We excluded one male from the experiments because after one week of acclimation it still had not eaten, therefore we released it to avoid any health problem. Six faecal samples were discarded because the amount was not enough for assay (2 white males and 4 yellow males), so our final sample was represented by 34 individuals (18 white males and 16 yellow males).

Hormonal treatment

After 4 treatment weeks, all males of both morphs showed a significant increase in faecal T concentration (β= 11.3 ± 4.5, *P* = 0.01). No difference between in morphs were observed (interaction morph x treatment *P* = 0.08): the treatment worked on both morphs and the yellow and white males increased their testosterone level.

Behavioural and immunological experiment

In the 95.5% (65 over 68 trials) of cases lizards approached the mirror, and bit the reflected image in 50.8% of cases (33 over 65 trials). The time spent in the half-mirrored cage ranged from 0 to 866 s, being on average 417 ± 274 s, lizards entered the half portion of the cage hosting the mirror on average 7.3 ± 4.7(range 0-19) times and the mean Tongue Flicking number was 43 ± 40 (0 – 196) (Tab. 1). Considering morphs, yellow males had mean, standard deviation and (range) of: Nmirror 7.1 ± 4.7 (range 1-19), Time 358 ± 265 (0-837), TF 39 ± 39 (0-160) and the mode of Bite 31 (interquartile distance 0-2); white morph, instead, have mean, standard deviation and (range) of: Nmirror 7.4 ± 4.8 (range 0-19), Time 469 ± 276 (1-866), TF 47 ± 40 (0-196) and the mode of Bites 7 (interquartile distance 0-13). Finally, average PHA_tot was 980 ± 869 (range 16-3550) for yellow males and 1276 ± 1236 (16-5136) for white males, and average PHA_cfu was 23 ± 19 (0-70) for yellow males and 32 ± 38 (0-187) for white males (Tab. 1).

The statistical analysis of behavioural experiments showed that Bites had a significant decrease in the post-treatment (β= -1.67 ± 0.55, P = 0.003). Contrary to what we expected, the increase in T level was not followed by an increase in aggressive response. The statistical analysis performed for Time, Nmirror and TF did not show any statistically significant effect of both Treatment and colour morph: the increase of T levels do not affect individual's space exploration (Tab. 2)

The statistical analysis of immunological experiments showed a decrease of PHA_cfu (β= -26.6 ± 9.22, *P* = 0.007) in both morphs regardless of size. The increase of T levels led to a reduction of immunoresponse. Our results did not show any statistically effect of Treatment over PHA_tot (Tab. 2).

Finally, the relation among the T levels, aggressive and immune response showed that Bites significantly varied depending on the treatment and the interaction between morph and both PHA_cfu and treatment. In detail, yellow males had a less number of colony-forming units ($\beta = -0.03 \pm 0.01$, $P = 0.024$) and also they decreased the aggressive response in the post-treatment ($\beta = -1.97 \pm 0.86$, $P = 0.022$). As ICHH stated, following a plasma T levels increase yellow males showed lower immunocompetence that reflected the reduced aggressiveness. However, the interaction between PHA_cfu x Treatment did not show any significant effect (Tab. 3).

DISCUSSION

In this paper we analysed the effect of plasma T levels manipulation over immunocompetence and aggressive behaviour, in two morphs of polymorphic lizards, *P. muralis*. We experimentally increased plasma T levels of males Common wall lizards and we measured before and after the hormonal treatment the immune response using cell culture with PHA inoculated and aggressive response using a mirror inserted in lizards' cage mimicking an intrusion of stranger. Our results show a decrease of colony-former unit (PHA_cfu) and a decrease of the number of bites against the mirrored image (Bites) in post-treatment. The decrease of immune response is due to the increase of plasma T levels, as stated by ICHH (Folstad & Karter, 1992; Oppliger et al., 2004), while the decrease of the aggressive response could not be attributable to a direct effect of testosterone (Moore & Marler, 1987), but rather to an effect due to a lower health condition (Marler & Moore, 1988). It was noticed in lizards' species a significant reduction in social behaviour, including aggressiveness, due to the increase of circulating corticosterone (Rada, Kellner & Winslow, 1976; Moore & Marler, 1987; Marler & Moore, 1988; DeNardo & Licht, 1993; Huyghe et al., 2009). Corticosterone is a hormone mediator to the effect of stress on intra- and intersexual reproductive behaviours (DeNardo & Licht, 1993). Thus, the immunosuppression leads to higher stress, modulated by corticosterone, which in turn inhibits aggressive response despite high T levels. Moreover, the results have shown a higher immunosuppression by yellow males, with a consequently lower aggressive response. This is in line with our hypothesis of presence of two strategies played by yellow and white males, and postulated by ICHH. In fact, males of this species showed a morph-specific pattern in immunocompetence, seasonal variation of plasma T levels and seasonal variation of aggressiveness (Sacchi et al., 2007a, 2014, 2017a,b; Coladonato et al., 2020). Therefore, yellow males invested more in aggressiveness, at the expense of better stamina, and we considered it as 'risky strategy'; instead white males focused on longer survival with less aggressiveness, and we referred to as a 'conservative strategy' (but see Coladonato et al., 2020). Given that, our experiments have shown that following a immunosuppression by a T levels manipulation, yellow males are more susceptible to stress variation and could easily be affected by diseases and parasites than white males, that is indeed genetically more adept at dealing with diseases and parasites (Andrade et al., 2019).

The effects of T levels manipulation on immune and aggressive response was widely studied over many lizards species (i.e.: Moore & Marler, 1987; Marler & Moore, 1988; DeNardo & Licht, 1993; Veiga et al., 1998; Olsson et al., 2000; Belliure, Smith & Sorci, 2004), even in *P. muralis* (Oppliger et al., 2004), but any work shown a morph-specific pattern response or correlated the increased testosterone with immunity and aggressiveness. Immunocompetence Handicap Hypothesis was already found in this species, but without considering morphs (Oppliger et al., 2004) and only tested immune response. In this paper we carried out, for the first time, an experiment to evaluate the morph-specific pattern in immune and aggressive response also taking into account the possible interaction between the health condition and the aggressiveness.

In Coladonato et al., (2020) we found that yellow males of Common wall lizards played a risky strategy involving more investment in aggressiveness despite the immune function. With this work we have seen this morphs is not able to deal with disease in higher T levels situation, hence we can confirm our previous hypothesis. The CP in this species could be maintained by a correlation between immune function and aggressiveness, as stated by ICHH.

	White	**Yellow**	**Total**
		Aggressive behaviour	
Bites*	7 (0-13)	31 (0-2)	15 (0-8)
	(0 - 74)	(0 - 59)	(0 - 74)
NMirror	7.4 ± 4.8	7.1 ± 4.7	7.3 ± 4.7
	(0-19)	(1-19)	(0-19)
Time	469 ± 276	358 ± 265	417 ± 274
	(1 - 866)	(0 - 837)	(0 - 866)
Tongue Flicking	47 ± 40	39 ± 39	43 ± 40
	(0 – 196)	(0 – 160)	(0 – 196)
		Immunity	
PHA_tot	1276 ± 1236	980 ± 869	1136 ±
	(16 - 5136)	(16 - 3550)	(16 - 5136)
PHA_cfu	32 ± 38	23 ± 19	27 ±
	(0 - 187)	(0 - 70)	(0 – 187)

Table 1. Mean (min – max) values of each response variable. Asterisk (*) is for Poisson distributed variables and the mode is indicated with the first and third quartiles. For all variables, the range is indicated (min.-max.) above.

	F/χ^2	**Df**	***P***
Testosterone Concentration			
Morph	1.96	1	0.16
Treatment	**13.9**	**1**	**<0.001**
SVL	1.2	1	0.27
Morph:treatment	0.06	1	0.80
Bites			
Morph	**9.15**	**2**	**0.01**
Treatment	**20.5**	**2**	**<0.001**
SVL	0.008	1	0.93
Morph:treatment	**23.36**	**3**	**<0.001**
Time			
Morph	2.43	1	0.13
Treatment	0.17	1	0.69
SVL	0.01	1	0.91
Morph:treatment	0.33	1	0.57
NMirror			
Morph	0.11	1	0.74
Treatment	0.09	1	0.76
SVL	0.66	1	0.42
Morph:treatment	0.14	1	0.71
Tongue Flicking			
Morph	0.68	1	0.41
Treatment	0.98	1	0.33
SVL	0.90	1	0.35
Morph:treatment	0.02	1	0.89
PHA_tot			
Morph	1.12	1	0.30
Treatment	0.30	1	0.58
SVL	0.07	1	0.80
Morph:treatment	0.76	1	0.39
PHA_cfu			
Morph	1.34	1	0.26
Treatment	**6.27**	**1**	**0.02**
SVL	0.11	1	0.74
Morph:treatment	2.11	1	0.16

Table 2. Effect of Morph, SVL, Treatment and the interaction Morph x Treatment on Testosterone concentration, Bites, Time, Nmirror, Tongue flicking, PHA_tot and PHA_cfu in males of Common wall lizards. Significant *P* values are reported in bold. For Bites are reported χ^2.

	Df	***χ^2***	***P***
SVL	1	0.07	0.79
PHA_cfu:Treatment	1	1.47	0.22
Treatment:Morph	**1**	**4.91**	**0.03**
PHA_cfu:Morph	1	3.38	0.06

Table 3. Results of interaction among PHA_cfu, treatment and morph. Significant P values are reported in bold.

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DISCUSSION & CONCLUSION

DISCUSSION

My PhD thesis aimed to investigate the presence of two distinct alternative strategies played by males of a polymorphic lizard species, the Common wall lizards (*Podarcis muralis*), and predicted by the ImmunoCompetence Handicap Hypothesis (ICHH, Folstad et al., 1993). Former studies on this lizard, have shown a morphspecific pattern for immunity and plasma Testosterone (T) levels throughout the breeding season: yellow males keep low stamina with high plasma T levels at the beginning of the season, while white males keep high stamina with a higher plasma T levels at the end of the season (Sacchi et al., 2007a; 2017b). Thus, I hypothesised that the yellow males played a ‘risky’ strategy as opposed to the strategy played by white males, considered 'conservative'. Testing the immune and the aggressive response, using both correlative and experimental approaches (**Chap. 4** and **5**), I found that white and yellows morphs actually seem to represent the two extremes of a trade-off between immunity and testosterone plasma levels. For the immune response I used well-known methods in scientific literature, such as cell culture *in vitro* (Oppliger et al., 2004; Sacchi et al., 2007a; 2014), but for the aggressive response I had to first solve some methodological limitations. In fact, former studies that tested the aggressive response supplied contrasting results (Sacchi et al., 2009; Abalos et al., 2016), and the effect of morphs on aggressiveness has not been clearly demonstrated. In order to assess how aggressiveness is triggered in *P. muralis*, the first part of my thesis focused to develop a reliable protocol for measuring the aggressive response in captivity (**Chap. 1, 2** and **3**).

In **Chap. 1** I proved that mirror, which joins different stimuli such as shape and motion, can trigger an aggressive response, contrary to what happens in other lacertid species for which shape is enough to release aggressiveness (i.e.: *Ctenophorus decresii*, *Platysaurus minor* and *P. monotropis*) (Yewers et al., 2016; Korner et al., 2000). This method allows removing the size’s effect because of the reflected image ”displays” the same size, and the same behaviour, of focal individual. Notably, focal individuals did not recognize themselves in the reflected image, and attacked it as a “true” intruder. Therefore, the intrusion by a stranger can be mimicked by inserting a mirror into the cage of a focal lizard. The method of this experiment is therefore can be reliably used to trigger the aggressive response in Common wall lizards and I used it to test aggressive response in all my other experiments in this thesis.

The main intrinsic problem of mirrors is that the image has the same colour morph of the focal individuals preventing the possibility to compare the aggressive response among morphs. The only way to investigate this aspect by using mirrors, is through the manipulation of color (**Chap. 2**). By this way it is possible to simulate the intrusion of a rival with a different morph with respect to the focal individual. In **Chap. 2** I manipulated the ventral colouration of lizards in order to obtain a mirrored image with a specific morph, so I was able to assess that males of Common wall lizards display a morph-specific pattern of aggressiveness. The individuals are often aggressive versus intruders of each morph, without difference in preferential aggression towards a specific morph, and the highest aggressive response was observed with intruders of the same colour morph. Morph-dependent aggressiveness allows males to minimize aggressive behavior, and therefore costs and risks, and triggered a more aggressive response against morphs that play the same strategy, since both strategies and morphs are genetically determined (Dijkstra et al., 2006; Yewers et al., 2016, Abalos et al., 2019). The reliability of lab results was tested by a field experiment, where the spatial distribution of morphs was analysed measuring the mean distance between morphs. The mean distance between neighboring individuals was significantly different when homomorphic and heteromorphic males were compared. These results suggest a non-random distribution with a repulsive effect of same-morph males. This uneven distribution, combined with the individual ability to recognise the scent marks produced by the femoral gland secretion, could build a spatial map of neighboring morphs. Decide which neighbors could exert a major threat to their territories and address aggression against their direct competitors, minimizing both the energetic costs of territory defence and the risks of suffering injuries or predation, according to the paradigm of the "dear-enemy effect" (Ydenberg, 1988; Whiting, 1999; Carazo et al., 2008; Tumulty, 2018). This experiment allowed me to affirm that the mirror protocol for measuring the aggressive response leads individuals to express the maximum aggressiveness.

The results of last experiment (**Chap. 3**), which concludes the first part of my thesis, explored how the housing conditions (namely, the acclimation in the cage) affect the interaction with the mirror, and, consequently, the measuring of aggressiveness. Data clearly show that lizards displayed a more intense aggressive response, and for a much longer time, when fighting for the territory of which they had a prior knowledge rather than for the territory not previously explored. Indeed, when switching from the neutral to the home arena, the interactions with the mirror image became longer and they involved a larger number of bites. The mirrored image, that mimicks a intruder, removes any asymmetry in Resource-Holding Potential (RHP) (i.e., the ability to gain and hold the disputed resource, Parker et al., 1974) and the encounters that occur between males with the same objective Resource Value (RV), where the only differences were the subject's prior knowledge of the arena. This result supports the occurrence of a direct, positive, correlation between motivation and aggressive response in Common wall lizards. This work was another step necessary to clarify that the stimulus that triggers aggressiveness is given by the resource value, i.e. their own territory, and with the mirror protocol the encounters take place in context where the aggressiveness is allowed to express at the maximum level.

By using mirrors in **Chap. 4**, I was able to measure the opposite seasonal patterns of aggressiveness displayed by white and yellow males, which supported (at a correlative level) the occurrence of morph-specific patterns of response. Yellow males showed a higher level of aggressiveness (number of bites against their mirrored image) compared to white males in the first part of breeding season, but this difference reversed as the season progressed. Having said that, the experiment measured the aggressive response in the most stimulating context (regarding RHP and morph-specific aggressive pattern, but see **Chap. 2** and **3**), the experiment allows to consider my results as the maximum possible aggressive response in a given period. These results follow the morph-specific seasonal pattern in plasma T level (Sacchi et al., 2017b), and support my prior hypothesis of the presence of two alternative strategies modulated by ICHH trade-off. Since the aggressiveness has an inverse correlation with stamina, these alternative strategies come out by a trade-off between two contrasting needs: territorialism, i.e. aggressive behavior, which allows individuals to defend resources for example food sources and more females, and longer survival. By keeping lower aggressiveness early in the season, white males are able to increase their chances of survival by reducing the risk of injury or by decreasing the immunosuppression due to stress. Yellow males, on the other hand, to achieve fitness concentrate all fight efforts in the early season, at the expense of survival. Different strategies played by different morphs can help individuals to recognize the strategy of rivals and modulate their own, avoiding conflicts against individuals who do not contend with the same resource at that time.

Finally, to experimentally demonstrate that the morphs are two opposite settings of the ICHH trade-off, I manipulated experimentally plasma T levels of males (**Chap. 5**). The results obtained highlight no difference in the increase of T between morphs: males increase T levels independently of morph. Hormonal treatment was able to break immune system with the decrease of colony-former unit in the post-trial, and this agrees with the prediction of ICHH. Contrary to what one might expect from the T-increase, I obtained a lower aggressive response. Health condition leads the animal behavior, therefore when animals are stressed, they are less likely to have high aggressiveness (Marler & Moore, 1988). Taking into account the interaction between the health condition and the aggressiveness, it can be seen that the highest immunosuppression, therefore lowest aggressive response, occurred in yellow males. Thus, this morph, which was genetically determinate as well as its immunodepression, is not able to deal with diseases and parasites and it is more susceptible to stress variation (in this case, the increase of T levels). At the end, yellow morph plays a strategy that maximizes the expression of sexual behaviour traits, with higher aggressiveness early in the season, while white morph aims to the ability to resist from parasites and diseases. Together, represent opposite settings of ICHH trade-off.

CONCLUDING REMARKS

This thesis confirms the presence of two alternative strategies, played by yellow and white males: the former shows a higher aggressiveness in the early season, but its immune-system is not able to deal with diseases and seems more susceptible to stress variation; the latter shows a higher aggressiveness at the end of the season, and its immune-system has lower decrease after manipulation of plasma T levels. CP could be maintained also by a correlation between immune function and aggressiveness.

Last, but not the least, with my work, especially **Chap. 1, 2** and **3**, I developed a protocol for measuring aggressive response which reflects the most stimulating context, regard with RHP and morph-specific aggressive pattern, putting the males in the condition of expressing maximum aggressiveness. This protocol could be replicated in other lacertid species.

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