



Original Article

# A chorus of color: hierarchical and graded information content of rapid color change signals in chameleons

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Received 22 September 2017; revised 19 March 2018; editorial decision 18 April 2018; accepted 17 May 2018.

Animals rely on information-rich signals to minimize costs associated with competition. If fighting ability is linked to stable individual attributes (e.g., morphology), the signals that communicate information about such ability should be relatively static. Conversely, the temporal variability of motivation should favor dynamic threat signals that indicate an animal's current likelihood of escalating a contest. Though static color ornaments are used by many animals to signal quality or fighting ability, the function of dynamic color change as a social signal has only recently begun to be investigated. Here, we examined the information content of rapid physiological color changes displayed by adult male veiled chameleons *Chamaeleo calypttratus* during agonistic interactions by conducting experimental trials between live chameleons and standardized, experimentally controlled robochameleon models. Chameleons reliably communicated motivation with dynamic color displays—individuals that brightened were 14 times more likely to approach the robochameleon than nonbrightening individuals. Additionally, chameleons with shorter latencies to maximum stripe brightness had stronger bites, and those displaying brighter, yellower stripes exhibited more aggression. The parallels between dynamic color changes and the vocalizations used to mediate aggressive interactions in other taxa are numerous. The use of particular vocalizations/color changes can signal motivation levels while specific signal elements (e.g., pitch, timing, brightness) may be linked to fighting ability. Because the complexity and potential information content of color signals increases markedly when organisms can display context-specific variation in the expression of these ornaments, the study of dynamic color signals is a field ripe for the investigation of complex visual and signaling strategies.

**Key words:** animal communication, chameleons, dynamic signals, honest signals, performance, physiological color change, robots, song, visual signals.

## INTRODUCTION

Throughout the animal kingdom, individuals engage in competitive interactions over indivisible resources (Hardy and Briffa 2013). In the context of competition, selection should favor the production and assessment of signals that convey information about both the relative value of contested resources [often referred to as motivation (Parker and Stuart 1976; Enquist 1985)] and resource holding potential [fighting ability (Parker 1974)] of contest participants because such signals can expedite aggressive interactions and reduce unnecessary costs associated with asymmetric conflicts (Parker 1974; Enquist 1985; Maynard Smith and Harper 2003; Searcy and Nowicki 2005). Specifically, signals that allow contest

participants to recognize large asymmetries in motivation or fighting ability enable individuals to save time, reduce energy expenditure, and resolve conflicts without the risk of physical violence (Rohwer 1982; Searcy and Nowicki 2005).

By their nature, static signals can only convey information about stable attributes of fighting ability whereas short-term motivational information is best communicated through dynamic displays (Enquist 1985; Bradbury and Vehrencamp 1998; Hurd and Enquist 2001). Dynamic signals of aggression may provide information about motivation via repertoires of distinct threat displays which can provide information about discrete escalation stages (Bradbury and Vehrencamp 1998; Enquist et al. 1998), or through graded signals which can provide graded information about motivation or aggression (Maynard Smith 1982). Discrete hierarchical signals of strategy are frequently evaluated in terms of whether a given behavior is displayed or not-displayed and how this choice relates to the subsequent aggressive behavior exhibited [e.g., the soft songs,

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matching songs, and wing-waving of song sparrows (Vehrencamp 2001; Ballentine et al. 2008; Akçay et al. 2013; Searcy et al. 2014)]. Alternatively, graded signals of motivation are scored along a continuous axis and are evaluated based on the relationship between signal intensity and overall aggression [e.g., call duration in frogs (Wells and Schwartz 1984; Schwartz 1989; Grafe 1995), song diversity in banded wrens (Molles 2006; Vehrencamp et al. 2014)]. However, some signals may fit both definitions if their display (or lack thereof) is informative *and* if there is subsequent variation among individuals that exhibit the behavior which is linked to additional variation in aggression (Akçay et al. 2014).

Rapid color change, which can serve as an agonistic signal, represents an interesting case study regarding links between signal design and information content because it possesses both static and dynamic attributes (Bagnara and Hadley 1973; Nilsson Sköld et al. 2013; Hutton et al. 2015; Ligon and McCartney 2016). Unlike fixed ornamental colors (e.g., of hair, feathers), physiological color change allows individuals to display different colors in changing environmental and behavioral contexts (Adamo and Hanlon 1996; Cuadrado 2000; Stuart-Fox and Moussalli 2008; Stuart-fox et al. 2008; Umbers 2011; Ligon 2014). This plasticity suggests that certain color change signals may have more in common with behavioral displays than with static colors. In contrast, the hues and patterns displayed by color-changing organisms are influenced by previously created structural elements or previously deposited pigments (Bagnara and Hadley 1973; Cooper and Greenberg 1992; Teyssier et al. 2015), which suggests that these signals also have a great deal in common with static color signals.

The temporal flexibility of rapid color change has the potential to increase signal complexity and thereby the number of potentially informative characters associated with these traits (Stuart-Fox and Moussalli 2009; Batabyal and Thaker 2017). Despite the potential for elements of complex color change to contain different types of information, most of the intraspecific color-change signals studied to date are somewhat simplified and correspond, roughly to a two-category hierarchy of aggressive signals (on = aggressive, off = not aggressive). For example, cephalopods (Adamo and Hanlon 1996), salmonid fish (O'Connor et al. 1999; Höglund et al. 2000; Eaton and Sloman 2011), cichlids (Muske and Fernald 1987), and *Anolis* lizards [Goldman and Hadley 1969; Summers and Greenberg 1994; Korzan et al. 2006] all seem to rely on simplified on/off color change signals that indicate aggressive intent or lack thereof. Though dynamic color displays could potentially provide more information than simple contest strategy (Bagnara and Hadley 1973; Nilsson Sköld et al. 2013; Hutton et al. 2015; Ligon and McCartney 2016), no subtle relationships have been uncovered between physiological color change and the various aspects of individual condition (e.g., fat reserves, immune function, body condition, strength) repeatedly discovered in taxa displaying fixed color signals.

Recently, we demonstrated that different aspects of dynamic color change in male veiled chameleons *Chamaeleo calyptratus* are correlated with the likelihood that a chameleon would approach his opponent and would win an aggressive interaction (Ligon and McGraw 2013). However, we still do not know how particular aspects of these color changes or other potential predictors of contest outcomes (e.g., body size, hormones) are linked to motivation and fighting ability. Identifying the underlying mechanisms connecting color change to aggression and outcome will inform our understanding of the processes ensuring signal honesty, as well as our interpretation of the evolutionary trajectories linking contest-relevant information to specific signals.

To address these questions, we conducted experimental trials between adult male chameleons and standardized, remotely controlled, artificial chameleon opponents. We then used a model-averaging statistical approach to investigate potential links between display colorimetrics, behavior, morphology, physiology, and physical performance. Because lizards primarily inflict damage upon rivals by biting, we hypothesized that chameleon color displays may communicate information about bite force. Strong positive links between bite force and dominance have been discovered for numerous lizard species (Lailvaux et al. 2004; Huyghe et al. 2005; Husak et al. 2006), and thus colorful signals that accurately convey information about individual bite force or testosterone-mediated traits should be particularly valuable for male chameleons engaged in aggressive contests. We hypothesized that chameleon color changes during aggressive interactions would be linked to testosterone as a potential intermediate mechanism connecting color, behavior, and performance. Specifically, we examined testosterone because it underlies seasonal (Klukowski and Nelson 1998), sexual (Hews et al. 2012), and species-specific (Hews et al. 2012) differences in aggression among lizards (Moore and Lindzey 1992). Additionally, color change occurs more slowly in castrated *Anolis* lizards, indicating an interactive role with the catecholamines directly linked to rapid color change in this species (Summers and Greenberg 1994). Testosterone levels are also lower in subordinate males than in dominant males (Greenberg and Crews 1990) in this species, and these combined lines of evidence led us to hypothesize that testosterone may mediate conflict dynamics in chameleons both through actions on muscle mass and motivation, as well as an interactive influence on dynamic color change. A corollary prediction is that brightening chameleons would be more aggressive than nonbrightening individuals, and that elements of rapid color changes would be linked to variation in overall aggression among brightening chameleons (Ligon and McGraw 2013).

## MATERIALS AND METHODS

### Study species and housing

Veiled chameleons are territorial lizards native to the southwestern Arabian Peninsula (Nečas 1999). Males regularly exhibit high levels of aggression towards conspecific males and use rapid color change to communicate during intraspecific interactions (Nečas 1999; Kelso and Verrell 2002; Ligon and McGraw 2013; Ligon 2014). During aggressive encounters, males also typically change their body shape and orientation, as they compress their bodies laterally while simultaneously undergoing dorsal-ventral expansion: effectively turning their bodies into billboard signs. At any time during the interaction, either male can cease aggression or, if threatened, rapidly retreat. Submissive behavior is accompanied by rapid darkening (Ligon 2014), but if both chameleons continue to exhibit aggressive behavior, contests can escalate to physical fights that include lunging and biting.

The veiled chameleons in this study were obtained from a private breeder and a feral population, both located in Florida, USA. Our chameleons were housed individually in a temperature-controlled vivarium at Arizona State University. Each cage contained a mixture of live, dead, and artificial plants to provide climbing structure and shelter, and was misted four times per day to provide drinking water for the chameleons. Additionally, each cage was fitted with a UV light source (Zoo Med Reptisun 5.0 UVB Fluorescent Bulbs; Zoo Med Laboratories Inc., San Luis Obispo, CA) and heat lamp

(Zoo Med Repti-Basking Spot Lamp, 50 watt). Additional details regarding chameleon housing and basic husbandry can be found in (McCartney et al. 2014).

### Behavioral Trials

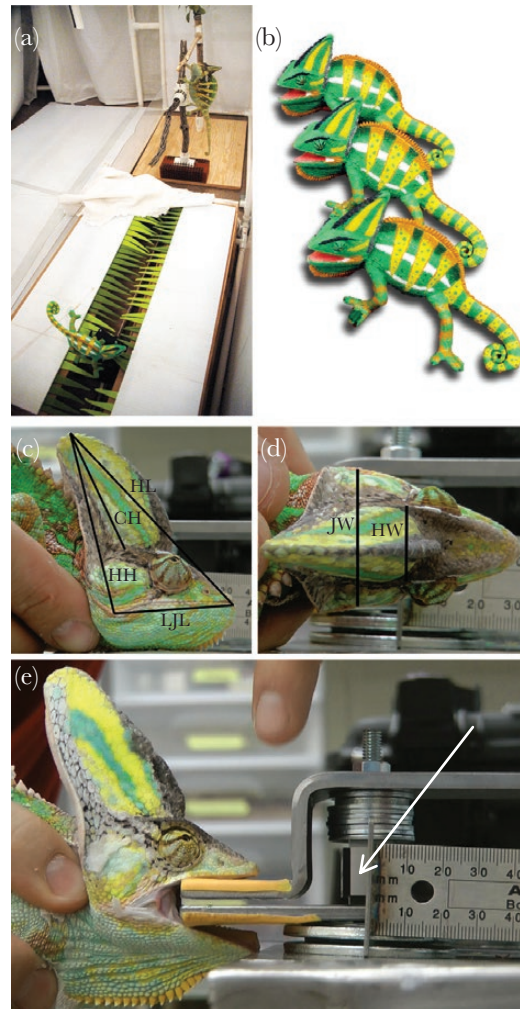
Over the course of 3 days (30 June–2 July 2013), we conducted aggression trials using 33 adult male veiled chameleons and life-like chameleon models (see *Robochameleons* section below). Because contest strategies are strongly influenced by opponent behavior, a standardized robotic model better enabled us to analyze the information content of the focal animal's signals without the confounding effects of opponent behavior (Ord et al. 2002; Martins et al. 2005; Klein et al. 2012). All chameleons in this study were longer [minimum snout–vent length (SVL) = 17.90 cm] than the robochameleon models (SVL = 12.50 cm), and each trial was conducted between one chameleon and one robochameleon. Prior to a given trial, we removed the test male from his cage, measured his body mass using a digital scale (accurate to the nearest 1 g), and placed him on one end of a trial arena (183 × 53 × 81 cm) containing vertical and horizontal perches (Figure 1a). During the subsequent 5-min acclimation period, the chameleon was visually isolated from the robotic chameleon model by a physical divider in the center of the arena. After acclimation, the divider was removed and the trial began. Trials were recorded from behind a blind with a Panasonic HDC-TM 700 video camera (Osaka, Japan), which enabled us to take still photographs while recording video. Trials were stopped after 10 min or after the chameleon physically attacked (i.e., lunged at and bit) the model more than once.

We used a customized version of the behavior logging program CowLog (Hänninen and Pastell 2009) to quantify every instance of 11 aggressive behaviors exhibited by chameleons during each trial (Supplementary Table 1). The sum of all instances of these aggressive behaviors served as the aggression score for each chameleon during each aggressive interaction.

### Robochameleons

We modified three commercially available plastic male veiled chameleon models (Safari Ltd®, Miami Gardens, FL) to create standardized, species-specific stimuli (Figure 1b) during trial presentations to live chameleons. Specifically, we removed the projecting tongue from each model and applied custom, nontoxic paints (Golden Artist Colors Inc, New Berlin, NY) created to mimic natural display coloration of veiled chameleons (Supplementary Figure 1). These custom paints were measured with a reflectance spectrometer (Ocean Optics, Dunedin, FL) and compared to representative spectra collected from live, displaying veiled chameleons using visual models (Vorobyev and Osorio 1998) incorporating chameleon visual sensitivities (Bowmaker et al. 2005). Differences between paint colors and live chameleon colors were determined in units of chameleon discriminability [measured in Just Noticeable Differences (JNDs) (Wyszecki and Stiles 1982)]. Our custom paints were similar to, but distinguishable from, chameleon coloration in both chromatic and achromatic channels (contrasts between paint color and chameleon color ranged from 0.80 to 5.57 chromatic JNDs and from 1.03 to 4.01 achromatic JNDs).

We animated our chameleon models by attaching them to a TrackerPod® (Eagletron Inc, Niagara Falls, New York) panning/tilting base designed for webcams. We controlled the TrackerPod® via a USB cord attached to a laptop computer that we placed near the trial arena. Additionally, we glued our TrackerPod® to a small,



**Figure 1**

(a) Experimental trial arena with live chameleon displaying towards robochameleon. (b) Custom-painted plastic veiled chameleon models used during behavioral trials. Custom paints were designed to mimic actual veiled chameleon display coloration, as seen by veiled chameleons (i.e., using visual models). (c) Morphological measurements taken from the head of each male veiled chameleon after bite-force measurements had been collected included: LJL, HH, HL, CH, and CW (measurement is not shown). (d) Additionally, we measured JW and HW. (e) Bite plates and load cell force transducer (arrow) set up to measure chameleon bite force. Yellow rubber on bite plates provides a compressible surface which prevents damage to the chameleons' teeth.

wheeled base and used a series of pulleys and string to control the forward/backward movement of the model during each trial. One individual (RAL) controlled all movements of each robochameleon during trials from behind a blind, observing the chameleon and robochameleon behavior via the LCD screen of one of our video cameras. The robochameleon began each trial facing away from the live chameleon, was slowly rotated to mimic the body orientation that typically follows identification of a chameleon opponent, and then was slowly advanced towards the live chameleon. To mimic the lateral display behaviors of live chameleons, wherein individuals orient their bodies perpendicularly to the direction of their opponent and sway, we stopped the robochameleon from approaching the live chameleons at short intervals and turned its bodies perpendicularly to sway.



When using dynamic artificial stimuli to elicit behavioral responses from live animals, one experimental approach is to employ an identical sequence of stimulus behaviors for every focal animal. We did not use this approach. Instead, we employed a protocol where the intensity of robochameleon behavioral responses roughly matched those of the live chameleon to maximize the likelihood that a given chameleon would respond aggressively to the robochameleon model and undergo physiological color change. This approach resulted in 13 of 33 chameleons (39%) undergoing aggressive color change in response to the robochameleon model, a result consistent with earlier behavioral studies between two live chameleons (e.g., aggressive color change in 34 out of 79 trials the preceding summer = 43%). The order in which the robochameleon models were presented was systematically varied, and the likelihood that a chameleon would approach (generalized linear model, GLM, with binomial error distribution; likelihood ratio test,  $\chi^2 = 5.73$ ,  $p = 0.06$ ) or brighten towards (GLM,  $\chi^2 = 1.78$ ,  $p = 0.41$ ) the robochameleon stimulus was not significantly influenced by robochameleon model identity.

### Morphological measurements

To measure SVL, one researcher used two hands to hold the chameleon in an outstretched position and a second researcher placed a flexible plastic ruler against the chameleon's body. Additionally, we collected seven measurements (Figure 1c,d) from the head region of each chameleon using digital calipers (accurate to the nearest 0.1 mm). Head measurements were chosen based on a previous investigation of the relationship between morphology and bite force in chameleons (Measey et al. 2009), as well as personal observations regarding a potential relationship between jaw and casque width (CW) and bite force. In total, we took morphometric measurements of head length (HL), head height (HH), casque height (CH), lower jaw length (LJL), head width (HW), CW, and jaw width (JW).

### Bite force performance

To assess potential links between bite force and chameleon display coloration, as well as morphology, testosterone, and aggressive behavior, we measured the bite force of each of our 33 chameleons 3–5 days before the behavioral trials. Bite force was measured three times for each chameleon and was highly repeatable [ $R = 0.55 \pm 0.10$ ,  $P < 0.0001$ , analyzed using package *rptR* in R (Stoffel et al. 2017)], though we chose to use maximum calculated bite force (see below) as a measure of individual biting power (Losos et al. 2002; Anderson et al. 2008). Only vigorous bites were recorded and we discarded those with abnormally low readings (Losos et al. 2002; Vanhooydonck et al. 2010). To quantify bite force, we used a miniature, low-profile load cell (Transducer Techniques®, Temecula, CA) fitted between custom bite plates coated with rubber (Figure 1d) to protect the chameleons' teeth when they bit down and to provide a compressible surface more similar to the biological matter (e.g., an opponent's flank) that they might typically bite.

We began each bite force measurement by placing a chameleon in front of the bite plates. Frequently, the chameleons would readily open their mouths as a threatening behavior (in response to being handled), and in these cases we simply placed the bite plates into their open mouths and waited for the animal to bite down. Other individuals opened their mouths when touched lightly around the head. Additionally, for others we had to manually open their mouths and place them onto the bite plates. To examine the

influence of our bite measurement protocol on bite force, including bite order and the amount of stimulation required to elicit bites from each chameleon, we used a linear mixed model created with the “lme4” package (Bates et al. 2014) in the R computing environment (R Core Team 2015). Our model included stimulation (scored as “handling,” “touching head,” or “mouth physically opened”), bite order (first, second, or third), and their interaction as fixed effects, as well as chameleon identity as a random effect. We interpreted the results of this model using the “afex” package (Singmann and Bolker 2014), also in R. Neither stimulation ( $F_{2,72.14} = 2.61$ ,  $P = 0.08$ ), bite order ( $F_{1,61.50} = 3.71$ ,  $P = 0.06$ ), nor their interaction ( $F_{2,69.30} = 1.71$ ,  $P = 0.19$ ) had a significant influence on bite force, though there were nonsignificant tendencies for bite force to increase with bite order and with increased stimulation level.

To correct for differences in mechanical advantage (i.e., force amplification), and thus measured output of bite force, arising from differences in the specific location where chameleons bit down on the bite plates, we used a high-definition video camera to record each series of bites in profile (Figure 1d). Video recordings included a metric ruler placed in the same plane as the bite plates, which allowed us to make measurements of the chameleon's head and bite location from still frames extracted from the videos of each bite event. Using the ruler within the extracted image to calibrate distance measurements, we were then able to measure the distance from the quadrate-articular jaw joint to the bite point (first point of contact between teeth and bite plate) using ImageJ (Schneider et al. 2012) which allowed us to calculate the true force applied by a chameleon's jaws using second order lever calculations [cf. (Lappin et al. 2006; Lappin and Jones 2014)].

### Testosterone measurement

We measured circulating testosterone levels of chameleons following agonistic interactions with robochameleons using blood samples collected immediately (<5 min) after each trial. Blood samples were collected following contests to minimize pre-trial stressors that might influence contest behavior. We collected blood samples from the caudal vein of each chameleon immediately after each behavioral trial using heparinized, 0.5 ml syringes. Blood samples were stored on ice until centrifugation, after which we froze plasma samples at  $-80^{\circ}\text{C}$  until analysis.

We measured plasma testosterone using commercially available enzyme-linked immunoassay (ELISA) kits (Enzo Life Sciences, Farmingdale, NY) in accordance with manufacturer's instructions. All samples for this study were run on a single ELISA plate that included positive and negative controls and standards to create a standard curve. Plasma T levels were calculated for each chameleon from absorbance values. All standards and samples were run in duplicate (mean intrasample coefficient of variation = 6.37). Additionally, the slopes of a plasma dilution curve created by serially diluting chameleon plasma ( $10\times$ – $100\times$ ) and that of the standard curve were statistically indistinguishable ( $F_{1,12} = 0.026$ ,  $P = 0.89$ ).

### Color measurement

We used digital photography to collect color and brightness data from chameleons during agonistic interactions with robochameleons following our previously published methods (Ligon and McGraw 2013). First, we analyzed video recordings of each aggression trial to determine visually the timing of rapid, agonistic color change bouts. We used photographs taken at approximately 4-s intervals during these color change bouts to quantify all color

and color change variables. Second, we standardized photographs [ensuring equalization and linearization (Stevens et al. 2007; Pike 2011)] using a specialized color standard (ColourChecker Passport, X-Rite Photo) and a software plug-in (PictoColour® inCamera™, PictoColour Software, Burnsville, MN) for Adobe Photoshop (Bergman and Beehner 2008). Third, we used specialized mapping functions (Pike 2011) to convert RGB (red, green, blue) values from standardized photographs to relative stimulation values of the chameleon photoreceptors (Bowmaker et al. 2005). Fourth, we plotted each color in chameleon-specific color space designed to preserve perceptual distances (Pike 2012). For full details, see [Supplementary Materials](#) in (Ligon and McGraw 2013).

We focused our skin color analyses on four patches on the head and lateral stripes; specifically, we chose two color patches on the vertical yellow body stripes and two locations on the heads ([Supplementary Figure 2](#)). These patches were chosen because their brightness and speed of color change were highly correlated with composite principal component (PC) scores collected from many, previously measured, locations within the same general body regions (Ligon and McGraw 2013) that predicted approach likelihood and fighting ability during male contests. For each color patch, we quantified the maximum brightness achieved [stimulation of chameleon double cones (Osorio and Vorobyev 2005)], the maximum speed of brightening, and the time it took (in seconds) to achieve maximum brightness from the beginning of the trial. We also measured color change, as the distance between the start and end color during brightening bouts calculated within chameleon color space (in units of JNDs), and the rate of color change (JND/s). Furthermore, we quantified maximum chroma for each color patch, and the hue at the point of maximum chromaticity. Maximum chroma was determined for each patch as the farthest point a given color travelled from the achromatic center of chameleon color space and hue was calculated as the angle of the vector connecting the achromatic center and a given color's location within color space at the time of maximum chromaticity (Endler and Mielke 2005; Stoddard and Prum 2008).

To reduce the number of variables in our analyses, we standardized each class of color measurements ( $x = 0$ ,  $SD = 1$ ) and averaged the colorimetric data for each body region (i.e., for the stripe region and for the head region). Thus, we created average values for brightness, maximum brightening speed, time to reach maximum brightness, color change (chromatic distance traveled), speed of color change (i.e., movement through chameleon color space/time), maximum chroma, and hue at maximum chroma for both the stripe and head regions.

## Statistical analyses

### Data preparation and investigation

Following prior recommendations (Zuur et al. 2010), we first evaluated our data for possible outliers using Cleveland plots, and for homogeneity of variance using plots of residuals versus fitted values for single predictor models representing subsets of the global models we evaluated. We assessed normality of residuals via visual inspection of Q-Q plots. Next, all variables were standardized to a mean of zero and a standard deviation of one (Schielzeth 2010) to facilitate direct comparisons of parameter estimates.

### Information-theoretic model-averaging approach

We used Akaike's information criterion corrected for small sample size (AICc) to evaluate all statistical models (Burnham and Anderson 2002). Specifically, we modeled relationships examining

continuous variables (testosterone, bite force) using linear models and relationships examining approach likelihood and aggressive behaviors using generalized linear models (approach behavior as a binary response variable with binomial distribution, aggression as count data with Poisson distribution). Overdispersion estimates from Poisson GLMs were within acceptable ranges [ $1 \leq \hat{c} \leq 4$ ; (Burnham and Anderson 2002)]. We evaluated our complete dataset ( $n = 33$ ) using models with three or fewer predictor variables [following rule of thumb described in (Harrell 2001; Bolker et al. 2009)]. Because not all chameleons underwent color change displays during aggressive interactions ( $n = 13$ ), thereby reducing our sample size, we limited our analysis of color change models to those with two or fewer predictor variables.

Though multiple models may be well-supported within an information-theoretic framework, evaluating the relative importance (RI) of specific variables is still possible using model-averaging approaches (Burnham and Anderson 2002; Burnham et al. 2010). Model averaging allows researchers to incorporate parameter estimates from multiple models, each weighted by the support for that model (Burnham and Anderson 2002). Thus, parameter estimates from well-supported models will contribute relatively more to multimodel parameter estimates. Information-theoretic approaches can generally provide accurate parameter estimates when confronted with collinearity among predictor variables, but we omitted additive models that included highly correlated variables ( $r > 0.7$ ) because of the increased variance among parameter estimates when models include highly correlated predictors (Freckleton 2010). Analyses of the variance inflated factors of our global models (as suggested by Burnham and Anderson 2002), which were generally high, confirmed the value of this approach, which limited the potential confounding influence of including models with highly correlated predictors.

The use of multiple models also allowed us to calculate RI values for each predictor variable within a given model set. Specifically, we calculated RI values by summing the Akaike weights ( $w_i$ ) for all models in which that variable appeared. Akaike weights for a given set of models sum to 1, so RI values range from 0 to 1 (where RI values near 0 indicate variables that occur infrequently or in poorly supported models and RI values near 1 indicate variables frequently represented in well-supported models). RI values were calculated from 95% confidence sets, for which the cumulative Akaike weight was  $\sim 0.95$ , indicating a 95% probability that the best model was within this set, or from the models that had lower AICc scores than the null model containing no predictor variables.

All statistical analyses were conducted within the R computing environment (R Core Team 2015). Additionally, model selection was performed using the MuMIn package in R (Barton 2013), and forest plots of parameter estimates and 95% confidence intervals using the Gmisc package in R (Gordon 2014).

### Animal ethics

All of the procedures described in this manuscript were approved by the Arizona State University Institutional Animal Care and Use Committee under protocol #10-1124R and comply with the laws of the United States.

## RESULTS

Over one-third (13/33) of the live chameleons that were allowed to interact with robotic chameleon models engaged in rapid brightening displays directed toward the robochameleon. Though body size

did not differ significantly between chameleons that brightened and those that did not (SVL, two sample  $t$ -test,  $t_{30,47} = -1.88$ ,  $P = 0.07$ , Cohen's  $D = 0.60$ ; mass, two sample  $t$ -test,  $t_{27,04} = -0.246$ ,  $P = 0.81$ , Cohen's  $D = 0.09$ ), chameleons that brightened were slightly, albeit nonsignificantly, longer than those that did not brighten.

Overall, there was a strong association between brightening and the likelihood of approaching the robotic chameleon opponent (GLM with binomial error distribution; likelihood ratio test,  $\chi^2 = 10.16$ ,  $P = 0.001$ , odds ratio = 14.40), indicating that brightening chameleons were 14 times more likely to approach the robochameleon than individuals that did not brighten. In the analyses described below, we evaluated relationships between morphology, testosterone, bite force, and behavior for all chameleons, but were forced to restrict colorimetric analyses to the subset of chameleons ( $n = 13$ ) that brightened in response to the standardized robotic chameleon.

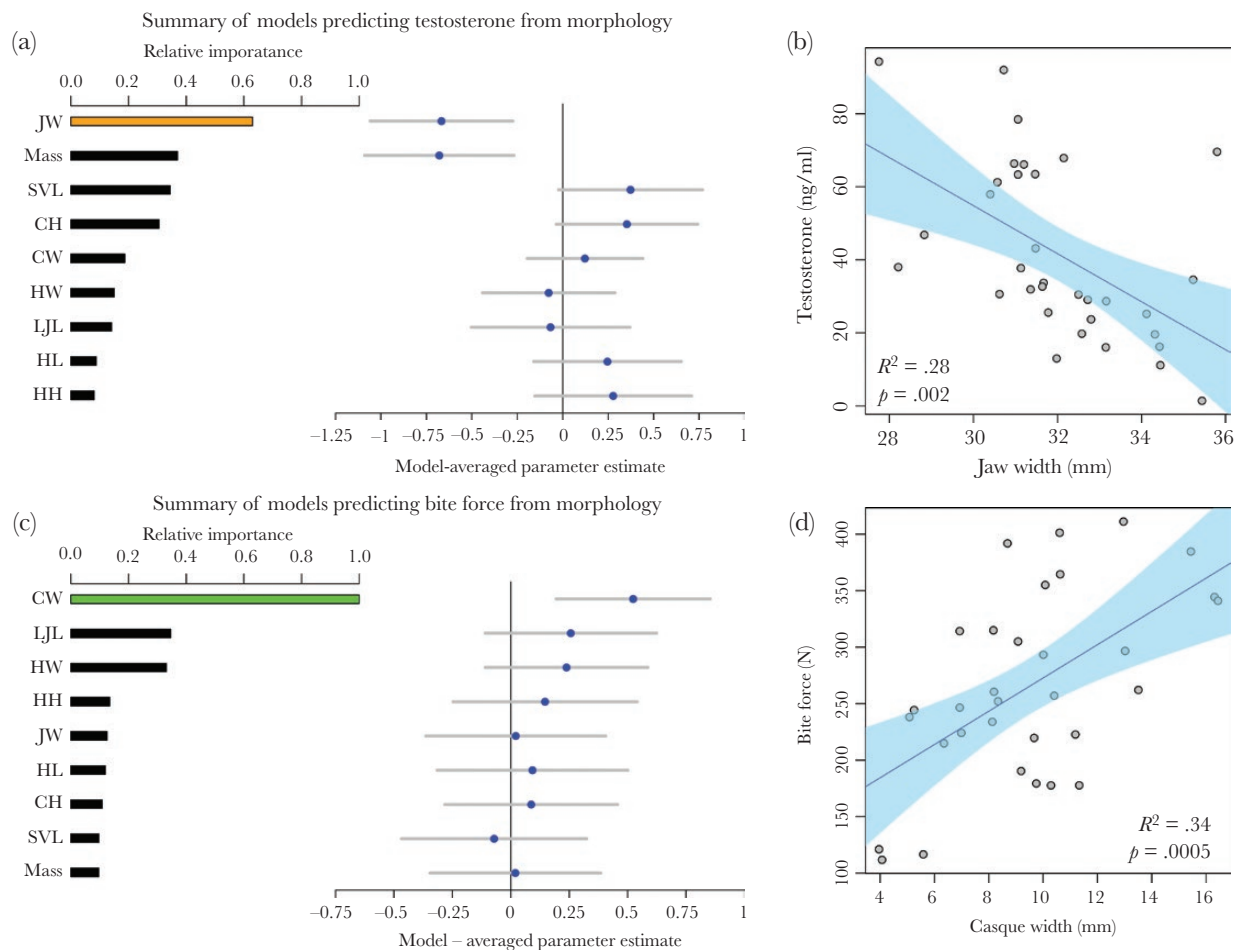
## All chameleons

### Morphological predictors of testosterone and bite force

Preliminary analysis of collinearity between morphological variables revealed strong correlations between multiple traits

(Supplementary Table 2). However, we omitted any models containing highly correlated ( $r > 0.7$ ) traits to minimize the effect that multicollinearity might have on the variance of parameter estimates. Evaluation of this set of models including only uncorrelated morphological predictor variables yielded no clear model as the best predictor of post-trial circulating testosterone concentration (Supplementary Table 3). However, multimodel averaging uncovered JW as the best predictor of circulating testosterone levels (RI = 0.63), with body mass (RI = 0.37) also being somewhat important (Figure 2a). Specifically, chameleons with narrower jaws ( $F_{1,31} = 12.08$ ,  $P = 0.001$ ,  $R^2 = 0.28$ ; Figure 2b) and smaller body masses ( $F_{1,31} = 10.23$ ,  $P = 0.003$ ,  $R^2 = 0.25$ ) had higher plasma testosterone levels.

Our analyses of the morphological predictors associated with maximum bite force yielded no clear best model (Supplementary Table 4). However, every model within our 95% confidence set contained CW as a predictor of bite force. Hence, CW (RI = 1.0) was by far the best predictor of the maximum bite force of male-veiled chameleons (Figure 2c); chameleons with wider casques had more forceful bites ( $F_{1,30} = 15.16$ ,  $P = 0.0005$ ,  $R^2 = 0.34$ ; Figure 2d).



**Figure 2**

Chameleon morphology is linked to testosterone and bite force. (a) RI values and parameter estimates of morphological variables predicting testosterone. (b) Relationship between testosterone and jaw width, the variable with the highest RI based on multimodel averaging. (c) RI values and parameter estimates of morphological variables predicting bite force. (d) Relationship between bite force and casque width, the variable with the highest RI based on multimodel averaging. Raw measurements are shown in (b) and (d) with 95% confidence intervals indicated by shading, but parameter estimates were obtained using standardized values. For (a) and (c), variables with RI values below 0.10 were excluded for clarity.

### Phenotypic characters (morphological variables, bite force, testosterone) and (i) likelihood of approach and (ii) peak aggression

Only a single model exploring the importance of phenotypic characters on the likelihood of approaching the robotic chameleon performed better than the null model (Supplementary Table 5). This model had SVL as the single predictor of approach likelihood, with longer chameleons exhibiting a nonsignificant tendency towards being more likely to approach the robotic chameleon (Figure 3a;  $z = 1.74$ ,  $P = 0.08$ , odds ratio = 2.29).

When examining the relationship between phenotypic characters and overall aggression, we identified two models whose cumulative raw Akaike weight was 0.996 (Supplementary Table 6). SVL was present in both models, giving it the highest possible relative importance (RI = 1.0; Figure 3b). Specifically, chameleons that were longer exhibited more aggression towards the robotic chameleon (GLM parameter estimate =  $0.63 \pm 0.08$ ,  $z = 8.33$ ,  $P < 0.0001$ ; Figure 3c).

### Brightening chameleons

#### Colorimetric predictors of testosterone and bite force

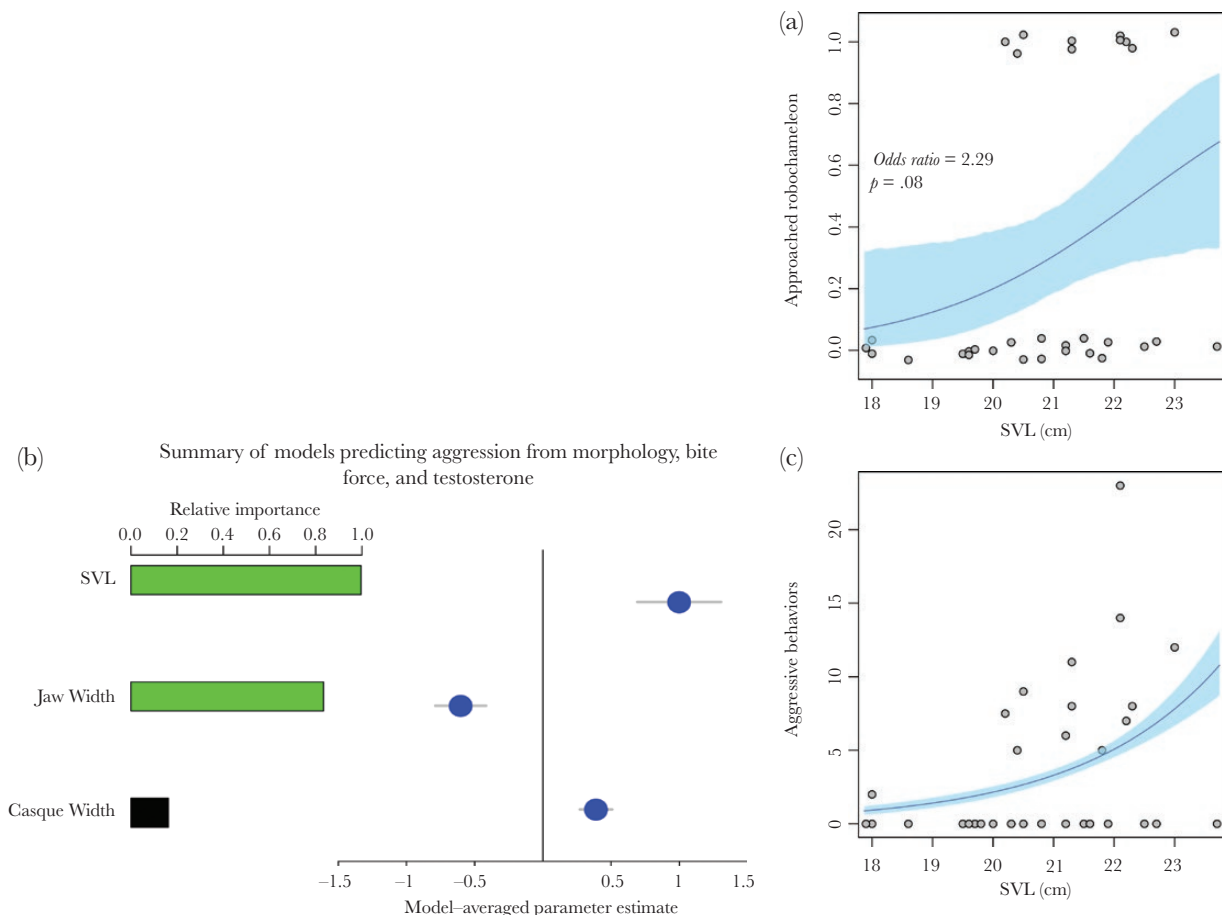
Among the 13 chameleons that brightened towards the robochameleon model, several color metrics were highly correlated

(Supplementary Table 7), so we restricted our models to those containing only uncorrelated variables. Two models linking color change and circulating testosterone performed better than the null model, both containing the maximum brightening speed as an explanatory variable (maximum stripe brightening speed RI = 1.0; Figure 4a, Supplementary Table 8). Chameleons that brightened more quickly had lower testosterone levels ( $F_{1,11} = 6.42$ ,  $P = 0.03$ ,  $R^2 = 0.37$ ; Figure 4b).

Analyzing the relationship between color metrics and bite force, we found that all 14 models that performed better than the null model contained the time to reach maximum stripe brightness as an explanatory variable (Supplementary Table 9; time to maximum stripe brightness RI = 1.0; Figure 4c); chameleons that reached maximum stripe brightness more quickly exhibited greater bite forces ( $F_{1,11} = 13.53$ ,  $p = 0.004$ ,  $R^2 = 0.55$ ; Figure 4d).

### Relative value of colorimetric and phenotypic (testosterone, bite force, and morphology) variables in predicting (i) likelihood of approach and (ii) aggressive behaviors

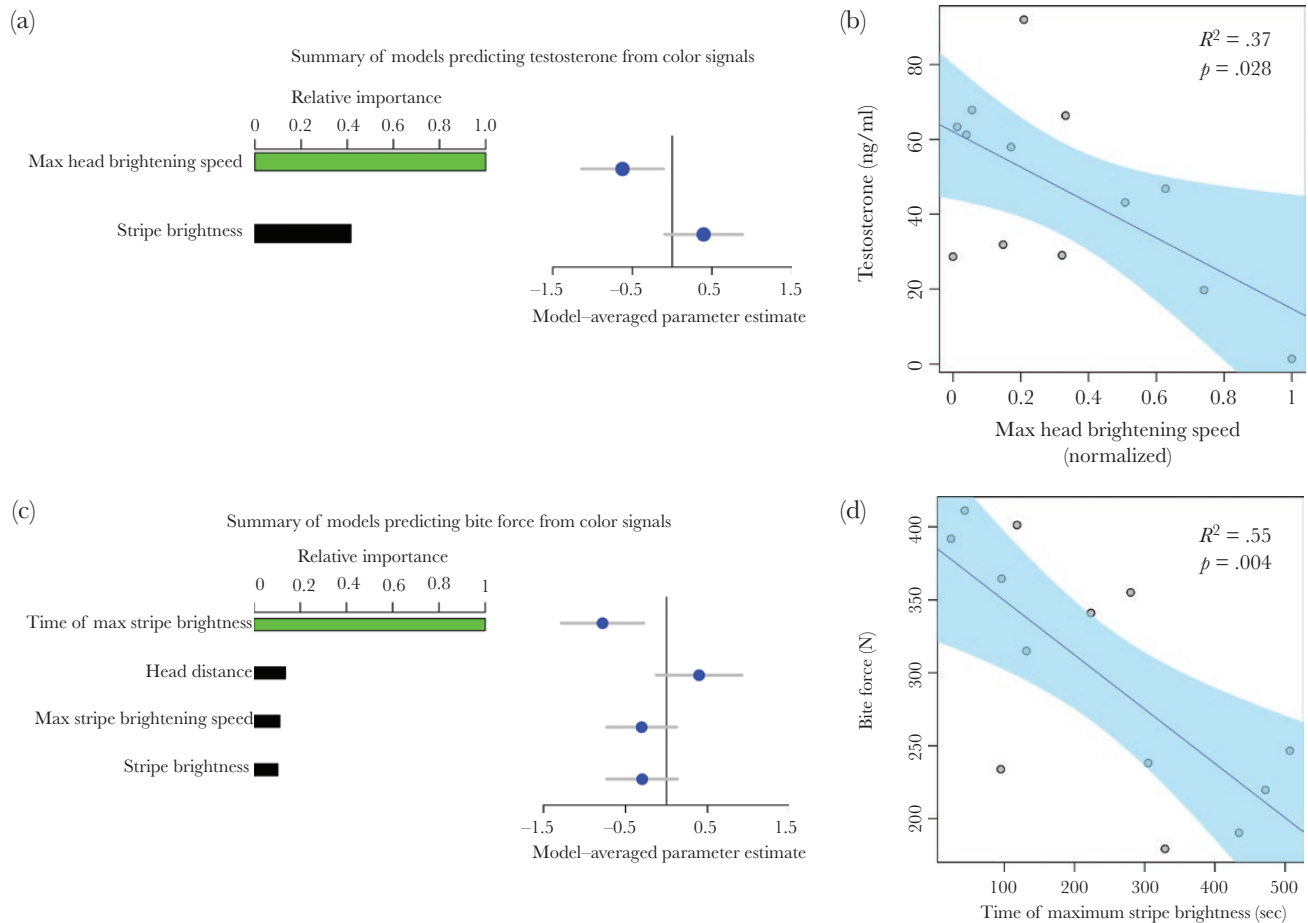
In an attempt to determine the RI of all color change and phenotypic variables in predicting the aggressive behavior of



**Figure 3**

Longer chameleons exhibit more aggression towards robochameleon models. (a) The likelihood of approach the robochameleon increased with an individual's SVL, the only variable in the single model that performed better than the null model. (b) RI values and parameter estimates of phenotypic characters predicting peak aggression displayed by chameleons toward robotic chameleons. Variables with RI values below 0.10 excluded for clarity. (c) Relationship between peak aggression score and SVL. Shaded regions in (a) and (c) represent confidence intervals based on  $10^5$  random samples from the multivariate normal distribution of model parameters. Raw values are shown in (a) and (c), but parameter estimates were obtained using standardized values.





**Figure 4**

Chameleons that brightened rapidly had lower testosterone, and those that brightened earlier had stronger bites. (a) RI values and parameter estimates of the color variables predicting testosterone. (b) Relationship between testosterone and the (normalized) maximum speed of chameleon head brightening. (c) RI values and parameter estimates of color change metrics predicting bite force. (d) Relationship between bite force and the time it took for a chameleon to reach maximum stripe brightness, the variable with the highest RI for predicting bite force, based on multimodel averaging. Normalized and raw numeric values shown (b and d, respectively), but parameter estimates were obtained using standardized values (95% confidence interval indicated by shading). For (a) and (c), variables with RI values below 0.10 were excluded for clarity.

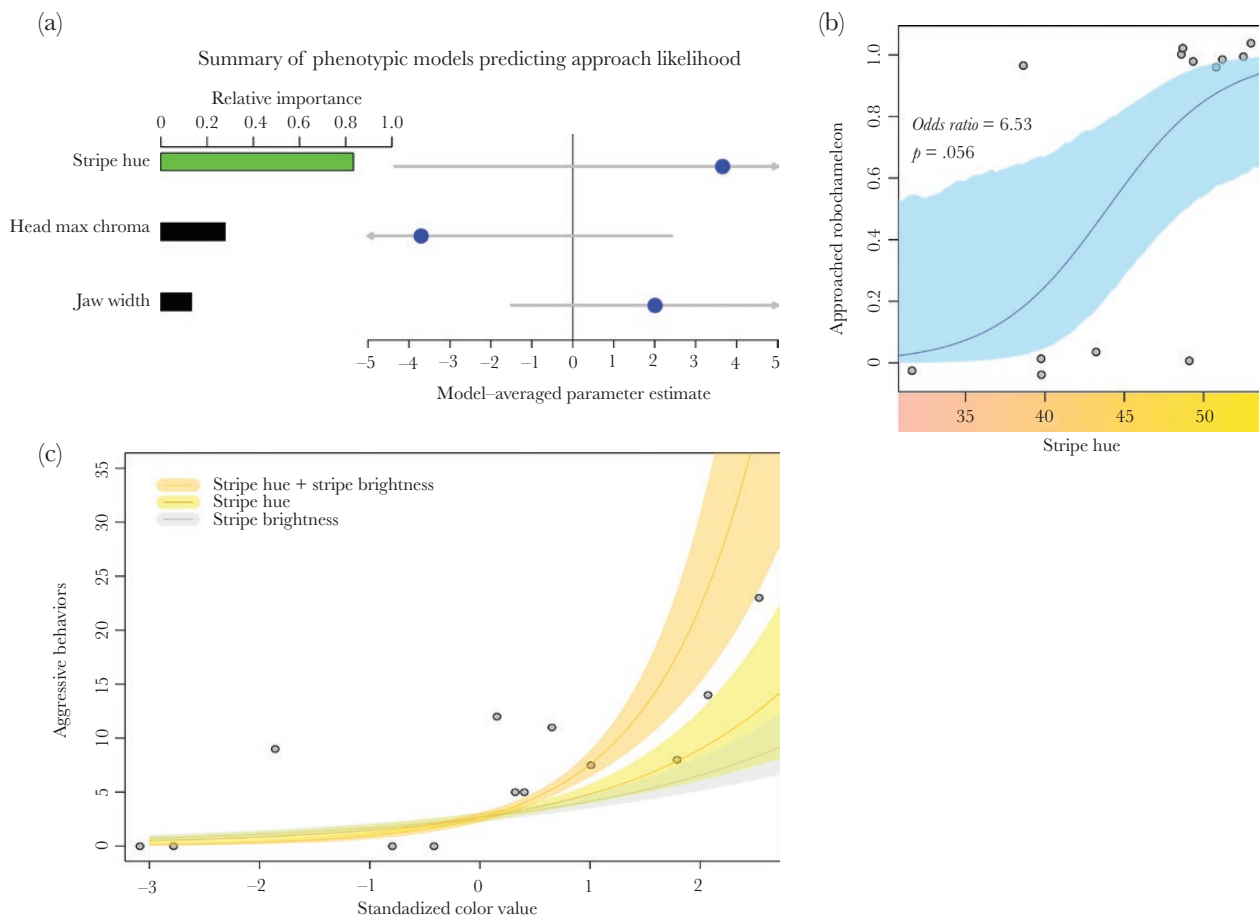
chameleons during agonistic trials with a robotic chameleon stimulus, we identified 25 models that performed better than the null model in predicting the likelihood of a chameleon approaching the robotic chameleon (Supplementary Table 10), and the variable with the highest RI was stripe hue (RI = 0.83; Figure 5a). This variable had a marginally significant influence on the likelihood that a chameleon would approach the robotic chameleon (GLM parameter estimate =  $1.88 \pm 0.98$ ,  $z = 1.91$ ,  $P = 0.06$ , odds ratio = 6.53; Figure 5b), such that chameleons with larger stripe hue values (more yellow, less orange) were more likely to approach the robotic chameleon. Additionally, we identified a single model containing stripe hue and stripe brightness that was, by far, the most well-supported model explaining the aggression displayed by chameleons towards the robotic chameleons (uncorrected  $w_i = 0.962$ ,  $\Delta\text{AIC}$  of next best model = 7.71; Supplementary Table 11). This model revealed that chameleons with yellower stripes (larger hue values) and brighter stripes exhibited higher levels of aggression (GLM hue estimate =  $0.60 \pm 0.13$ ,  $z = 4.50$ ,  $P < 0.0001$ ; GLM brightness estimate =  $0.45 \pm 0.07$ ,  $z = 6.23$ ,  $P < 0.0001$ ; Figure 5c).

## DISCUSSION

The dynamic color signals used by chameleons to mediate aggressive interactions are perhaps even more informative than previously suggested (Ligon and McGraw 2013; Ligon 2014). Namely, chameleons engaged in agonistic interactions can be placed into two discrete categories based on whether or not they brighten—brightening chameleons are much more likely to approach their opponents than nonbrightening chameleons. Hence, the decision to brighten during an aggressive encounter represents a discrete, two-step hierarchical signal of motivation, the honesty of which is maintained by social costs (Ligon and McGraw 2016). Additionally, variation in the timing and appearance of the displayed colors conveys additional, graded information about physical performance and motivation.

Contrary to our prediction that bite force, a putative proxy for fighting ability (Husak et al. 2006; Lailvaux and Irschick 2007), would be linked to long-term, relatively stable elements of the colorful displays used by veiled chameleons, we found that the time an individual reached maximum stripe brightness was the





**Figure 5**

Chameleons with yellow, brighter stripes were more aggressive towards robochameleon models. (a) RI values and parameter estimates of color change metrics and phenotype in predicting the likelihood of a chameleon approaching the robotic chameleon. Variables with RI values below 0.10 were excluded for clarity. (b) Relationship between approach likelihood and stripe hue, the variable with the highest RI based on multimodel averaging. Shaded region illustrates 95% confidence interval. Hue values were back-transformed to original measurements. (c) Relationship between aggressive behaviors and two standardized measures of stripe display coloration, stripe hue and stripe brightness. For the stripe hue and stripe brightness models, shaded regions represent confidence intervals based on  $10^5$  random samples from the multivariate normal distribution of model parameters with the other parameter held at mean empirical values (illustrating the exclusive influence of hue or brightness on aggression). For the stripe hue + stripe brightness model, the shaded region represents confidence intervals based on  $10^5$  samples from the multivariate normal distribution of model parameters where both variables varied, indicating the joint influence of these color variables on aggression (combined hue + brightness scores plotted).

best predictor of his bite force. The timing of maximum stripe brightness is inherently dynamic and, therefore, flexible—necessitating a change to the proposed “static signal indicates a static trait” model. One simple explanation is that bite force may be temporally labile (Irschick et al. 2006), favoring a dynamic signal communicating this attribute. Furthermore, if hard-biting chameleons fared better in previous aggressive interactions as a consequence of their biting ability, these winning experiences could partially account for the observed differences in latency to maximum brightness because prior success decreases latency to display and attack in a number of other species (Martinez et al. 1994; Adamo and Hoy 1995; Oyebile and Marler 2005). Additional, theoretically informed explanations can be found in game-theory models by Enquist (1985) and Enquist and Leimar (1983) exploring the dynamics of signaling strategies mediating contest behaviors.

Among brightening chameleons specifically, hard-biting individuals may signal fighting ability by quickly brightening when facing opponents (Figure 4d) because the strategy of an initial display of

strength maximizes contest pay-offs for strong individuals (Enquist 1985). Additionally, a rapid display of maximal signal information (e.g., maximum brightness) reduces ambiguity about the relative strength of contest participants, increasing the likelihood that opponents with lower fighting abilities should give up sooner (Enquist and Leimar 1983)—perhaps favoring reduced latency to maximum brightness among chameleons with large bite forces. On the other hand, individuals with lower biting strength may evaluate their opponent for greater time period before eventually signaling strength (i.e., brightening) because the costs of mis-signaling—signaling strength then giving up—are large if individuals end up being weaker than their opponents (Enquist 1985). However, it should be noted that “fighting ability” (measured as bite force) alone did not dictate display strategy (signaling strength vs. signaling weakness), because bite force did not differ between brightening and nonbrightening chameleons (two sample *t*-test,  $t_{30.03} = -1.18$ ,  $P = 0.25$ ). By never brightening, certain strong and weak individuals alike are indicating their nonaggressive intent and “satisfaction” with a net contest pay-off of 0.

In addition to the overall threat communicated by brightening *Per se* and the strategic implications of varying latency to maximum stripe brightness, the specific attributes of the colors displayed by brightening chameleons appear to represent graded signals containing additional, contest-specific information. Chameleons whose stripes were yellower and brighter (higher maximum brightness values) were more aggressive towards robotic chameleon models, a finding consistent with, though not identical to, our earlier work [we did not previously quantify hue (Ligon and McGraw 2013)]. Although bright, colorful displays have been linked to aggression and dominance in numerous other taxa [e.g., (Pryke and Andersson 2003; Siefferman and Hill 2005; Penteriani et al. 2007; Martín and López 2009; Steffen and McGraw 2009; Crothers et al. 2011)], the color signals explored to date have not been as temporally flexible as those displayed by chameleons. Because the pigments and structures responsible for producing a given color are exogenously obtained, endogenously created, and deposited well in advance of the contexts in which these colors are displayed, the chromatic signal elements used by chameleons (and other color-changing taxa) may be expected to reflect stable information about an individual's quality or diet [*sensu* (Hill et al. 2002; McGlothlin et al. 2007)]. However, the links we documented between hue, brightness, and aggression suggest that variation in the expression of underlying coloration serves as a graded signal of motivation. Much in the same way that a European starling's developmental conditions may influence its song repertoire size (Spencer et al. 2004), current conditions and context undoubtedly impact when songs are performed and for how long (both of which are linked to aggression (Gwinner et al. 2002)). Though diet and physiology undoubtedly influence the colors a given chameleon *can* display, the colors and patterns he *actually* displays appear to be influenced by, and communicate information about, current motivation.

The physiological characteristic which we predicted would be linked to aggression and motivation, testosterone, was not. Although testosterone may be an important mediator of aggressive behavior in some species at some time-scales, its short- (Smith and John-Alder 1999) and long-term (Klukowski and Nelson 1998) variability makes it difficult to predict its specific influence on contests or color change. Because we found no links between testosterone and aggressive behavior or bite force, suggesting that short-term variation in testosterone among males in our study population has no significant impact on contest dynamics, our ability to meaningfully interpret the observed negative relationship between testosterone and the maximum speed of head brightening is severely limited at this time.

In addition to evaluating the timing, color, and brightness of color changes exhibited by opponents during an aggressive interaction, veiled chameleons should also attend to particular morphological cues that could potentially provide contest-relevant information. Namely, CW, which is linked to bite force (Figure 2d), and body length, which is linked to aggression (Figure 3c). The relationship between bite force and CW makes sense when you consider that jaw musculature influences bite force (Lappin et al. 2006) and CW in veiled chameleons is directly linked to lateral jaw adductor musculature (R. Fisher, *pers. comm.*), which should enable greater bite force. However, the absence of a link between CH and bite force in veiled chameleons is worth considering, given that CH has previously been linked to bite force in the closed-habitat ecomorph of *B. pumilum* (Measey et al. 2009) and a small group ( $N=6$ ) of veiled chameleons (Vanhooydonck et al. 2007). Differences in casque morphology [e.g., shape, relative height, muscle attachment

points (Rieppel 1981)] are likely responsible for the observed differences between dwarf and veiled chameleons with respect to the relationship between CH and biting ability [see, in particular, ecomorphic and interspecific variation in the morphology-bite force links documented by (da Silva et al. 2014)], yet an explanation for the extreme CH of veiled chameleons (Hillenius 1966) is still required. We suggest that social selection has favored extreme casques in male veiled chameleons because such casques present a larger surface area for signaling via rapid color change. We have previously demonstrated that head-specific color changes are linked with fighting success in this species (Ligon and McGraw 2013), and larger casques may therefore provide more efficient or reliable means of communicating this information. Consistent with the idea that social selection pressures may favor the exaggeration of CH as a social signal in chameleons, rather than as a means of increasing bite force, casque size in male warty chameleons *Furcifer verrucosus* is four times more important for predicting mating success than fighting success (Karsten et al. 2009).

Differences in size, motivation, or fighting ability are predicted to influence contest behavior (Parker 1974; Austad 1983; Archer 1988). Thus, the direction of our results, where longer chameleons showed a trend towards being more likely to approach the robochameleon and were more aggressive towards it, are not terribly surprising. Body size can influence the likelihood of winning aggressive interactions for some lizard species (Aragón et al. 2005; Sacchi et al. 2009; Umbers et al. 2012), including two species of Madagascan chameleons (Karsten et al. 2009), and larger combatants are expected to incur reduced costs in agonistic encounters with smaller opponents (Austad 1983). Yet, for neither veiled chameleons (Ligon and McGraw 2013) nor Cape dwarf chameleons (Stuart-Fox et al. 2006) does body mass appear to be an important predictor of contest success. Bite force is not related to SVL ( $F_{1,30} = 0.23$ ,  $p = 0.64$ ) or body mass ( $F_{1,30} = 0.22$ ,  $p = 0.64$ ) in veiled chameleons; hence selection in competitive contexts is likely acting primarily on weapon performance (Lappin and Husak 2005) and signaling efficacy (Stuart-Fox and Moussalli 2008) rather than body size in this system. Interestingly, body mass and JW are both negatively correlated with testosterone, but given the lack of any demonstrated connection between testosterone and behavior or performance in the current study, attending to body mass or JW would not appear to provide any meaningful information to chameleons engaged in aggressive interactions.

## CONCLUSIONS

In this study, we uncovered links between morphology, physiology, performance, and aggression in male veiled chameleons, though stronger, more robust links were revealed between display colorimetrics and these same values. A veiled chameleon in an aggressive interaction with a conspecific can, therefore, acquire the most information about his opponent's physiological status, potential for inflicting injury, and motivation by attending to the rapid color changes of his opponent, but should also pay attention to the additional information conveyed by morphology.

Our findings provide new evidence that the information-content and complexity of the information conveyed via ornamental colors increases when rapid color change, brought about by the dynamic reorganization of pigmentary or structural components within the dermal chromatophore (Teyssier et al. 2015; Ligon and McCartney 2016), takes place within the time-frame of a given social interaction. The flexibility in signaling strategies facilitated by

rapid physiological color changes parallels that afforded to acoustically communicating animals like birds and frogs. Just as the presence or absence of song-type matching in song sparrows provides a two-stage, hierarchical signal of aggression (Akçay et al. 2013), the display (or lack of) rapid brightening by chameleons provides an unambiguous, discrete signal of motivation. Furthermore, variation among individual song sparrows in subsequent singing behavior provides additional information to opponents (Akçay et al. 2014), as does variation in the timing, brightness, and color of aggressively color-changing chameleons. Because the complexity and potential information content of signals increases markedly when organisms can display rapid, context-specific variation, the study of rapid color change signals is a ripe field for new explorations into the functions, mechanisms, and evolutionary origins of multicomponent signal types.

## SUPPLEMENTARY MATERIAL

Supplementary material can be found at *Behavioral Ecology* online.

## ACKNOWLEDGMENTS

We thank Kristen McCartney, Sarah Bruemmer, Megan Best, Brianna Bero-Buell, Andrea Carpenter, and Tierney Coates for assistance with chameleon husbandry, data collection, and photographic analyses. We thank Dan Huber, Kristopher Lappin, and Kristopher Karsten for advice regarding the construction of our bite plate setup, as well as James Makar for assistance in the bite plate construction. We thank Ellis Loew and Jim Bowmaker for providing the raw data necessary to complete our visual models. Matthew Toomey provided assistance in designing and implementing the chameleon visual models. We thank Martin Stevens and Thomas Pike for providing explanations of the processes required to calculate quantum catch using photographic methods. Thomas Pike also provided MATLAB code for estimating camera sensor sensitivities, determining photograph-to-cone catch conversions, and converting relative cone stimulation to Cartesian coordinates in perceptual distance/chromaticity space. We thank Scott Davies for invaluable assistance with testosterone measurement and Pierre Deviche for workspace in his lab. We thank Rick Simpson, Mary Ann Lee, Mike Webster, and Emma Grieg for detailed comments and suggestions on an earlier version of this manuscript. We thank the American Society of Ichthyologists and Herpetologists, the Animal Behavior Society, the American Society of Naturalists, and the Arizona State University Graduate and Professional Students Association for funding. RAL was supported by the ASU Graduate College Completion Fellowship and NSF Grants #1401236 and #1523895 during the writing of this manuscript. We also thank David and Sandy Ligon for early financial support, and Veronica Ligon for emotional support.

Data accessibility: Analyses reported in this article can be reproduced using the data provided by Ligon and McGraw (2018).

**Handling editor:** John Fitzpatrick

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