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Gonadal tissue color is not a reliable indicator of sex in rocky intertidal mussels

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ABSTRACT: Gonadal tissue coloration was previously thought to be a reliable indicator of sex (male vs. female) in intertidal mussels. However, no investigations have been performed to determine whether color is an accurate representation of sex and to evaluate how this relationship varies throughout the environment. Patterns of gonadal tissue coloration were examined in the mussel *Mytilus californianus* along 2 environmental axes during the summer of 2004: (1) a food-availability gradient across 4 sites on the central Oregon coast and (2) a vertical (tidal height) stress gradient within each of the sites. Gonadal tissue color was unrelated to food availability. Both male and female mussels at the high edge of the mussel bed had orange gonadal tissue, contrary to conventional wisdom that mussel sex can be determined visually by gonadal color (males = white, females = orange). Field classification based on tissue color is therefore an unreliable indicator of sex in mussels. Environmental stress appears to influence patterns of tissue color (carotenoid pigment concentration) in the intertidal zone.

KEY WORDS: Mussel · Carotenoids · Color · Environmental stress · Reproduction · Rocky intertidal zone

INTRODUCTION

To address ecological issues, such as energy allocation to reproduction or potential reproductive output of a population, it is often necessary to determine the sex of individuals. Visual identification of sex (male vs. female) based on gonadal tissue color is a common practice amongst field biologists studying a diversity of marine bivalve taxa (e.g. Jolley et al. 2004, Williams & Babcock 2005). Scallops, which are simultaneous hermaphrodites, are typically classified with the female portion of the gonad bright orange in color and the male portion white (e.g. Williams & Babcock 2005). Cockles and mussels, of which most species are gonochoric, are identified as male if gonadal tissue is white and female if gonadal tissue is orange (e.g. Jolley et al. 2004). In contrast, oysters are typically sequential hermaphrodites and have little obvious pigmentation in their gonadal tissues, making assignment of sex based on color difficult and histology the primary technique for determining sex (e.g. Lango-Reynoso et al. 2000). Despite the widespread use of color as an indicator of sex, coloration of gonadal tissue in marine invertebrates is complex and can vary with diet, season, age, and reproductive stage (e.g. Campbell 1969, Agatsuma et al. 2005). Therefore, the visual identification of sex based on color should be called into question.

For decades, ecologists have identified sex of mytilid mussels, competitive dominants for space on many temperate rocky shores, based on the color of the gonadal tissue. Individuals with white tissue are identified as males and mussels with orange tissue as females (Chipperfield 1953, Seed & Suchanek 1992), due to high concentrations of carotenoid pigments (Campbell 1969). Physiologists, in contrast, use histology as the primary method for examining reproductive health, gonadal stages, and seasonal patterns of maturity (e.g. Seed & Suchanek 1992, Alfaro et al.

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2001). In addition, microscopic examination of gonadal tissue with smears (e.g. Torrado & Mikhailov 1998) and a biochemical colorimetric analysis (Jabbar & Davies 1987) have been used to differentiate between sexes. However, no studies have attempted to integrate field observations of gonadal tissue color with actual sex as determined through histology. The validation of color as an indicator of sex in population ecology and aquaculture studies is critical for future usage of this technique for both intertidal mussels and other taxa.

Food availability may influence gonadal tissue color in mussels, given that mussels accumulate pigments from their phytoplankton food source (Campbell 1970). Two capes on the central Oregon coast differ in their oceanographic regimes and subtidal bottom topography, creating persistent differences in nearshore primary production (Menge et al. 2004, Leslie et al. 2005). Cape Perpetua has relatively high productivity, indicated by high chlorophyll *a* (chl *a*) concentration (Menge et al. 1997, 2004), which is a good estimate of phytoplankton abundance in nearshore marine systems (Menge et al. 1997). This high abundance of food leads to rapid growth rates of many filter-feeding invertebrates, including mussels (e.g. Menge et al. 2004). In contrast, Cape Foulweather (~63 km to the north) has relatively low phytoplankton abundance and, consequently, slower growth rates of sessile invertebrates (Menge et al. 1997, 2004).

Environmental stress, any characteristic of the physical environment causing changes in biochemical reactions by approaching or exceeding physiological tolerance limits of organisms (Menge & Sutherland 1976), could also influence tissue color. Stress can lead to a suite of negative consequences, including reduced growth, changes in metabolic rate, decreased fecundity, and mortality (e.g. Schreck et al. 2001). In the intertidal zone, stress increases along a vertical gradient, from the relatively low-stress low zone to the higher-stress high zone, as aerial emersion time increases with height on the shore (Davenport & Davenport 2005). Mussels span this vertical stress gradient, from the upper edge of the low zone to the lower edge of the high zone. Mussels at the high edge of the mussel bed live close to or at their physiological tolerance limits and can exhibit lethal (e.g. Petes et al. 2007) or sublethal (e.g. Helmuth & Hofmann 2001) signs of stress. Carotenoid pigments, which are responsible for the orange coloration of mussel gonads (Campbell 1970, Petes et al. in press), are known for their antioxidant properties (e.g. Miki 1991). It is therefore possible that there is a relationship between zonation and gonadal tissue color, because oxidative stress increases with tidal height.

Gonadal tissue color needs to be verified as an accurate method for assigning sex in mussels, and the relationship between food and stress on tissue color needs to be determined. The purpose of the present study was to test the following 3 hypotheses in intertidal mussels: (1) gonadal tissue color is a reliable indicator of sex in intertidal mussels; (2) food availability affects coloration of gonadal tissue; (3) environmental stress influences gonadal tissue coloration.

MATERIALS AND METHODS

To determine if gonadal tissue color is a reliable indicator of stress and to investigate how food and stress influence patterns of tissue color, mussels *Mytilus californianus* were collected monthly from May to September 2004 at 4 sites on the central Oregon coast (Fig. 1). Fogarty Creek (FC; 44.84° N, 124.06° W) and Boiler Bay (BB; 44.83° N, 124.06° W) are located at Cape Foulweather, and Yachats Beach (YB; 44.32° N, 124.12° W) and Strawberry Hill (SH; 44.25° N, 124.12° W) are located at Cape Perpetua.

Chl *a* and temperature measurements. Phytoplankton concentration was estimated using chl *a* to quantify the food source for mussels. Surf-zone chl *a* measurements were collected monthly from May to August 2004 on the same day at all 4 sites, as described by Menge et al. (2004). Briefly, replicate bottle samples (n = 3) of water were taken from shore and filtered in the field through combusted Whatman GF/F glass-

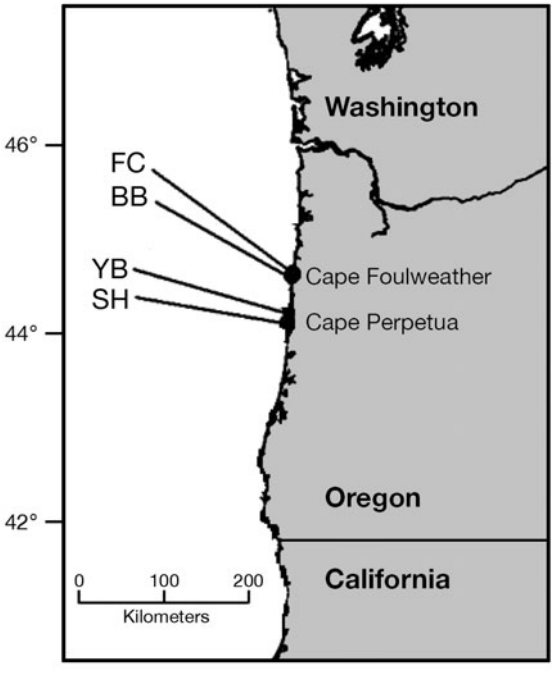

Fig. 1. Map depicting field sites on the central Oregon coast. FC: Fogarty Creek; BB: Boiler Bay; YB: Yachats Beach; SH: Strawberry Hill

fiber filters. Chl *a* concentration was determined using a Turner Designs TD-700 fluorometer (Turner Designs) according to Welshmeyer (1994). The fluorometer was calibrated with a chl *a* standard from Sigma Chemical Company.

One TidbiT temperature logger (Onset Computer Corp.) was deployed on bare substrate in the middle of the mussel bed at each site to record temperature (aerial or aquatic depending on tidal cycle) every 10 min from 1 May to 30 September 2004. At SH, 2 loggers were deployed from 1 April to 30 September 2004 to quantify differences in substrate temperature between the middle and the lower edges of the mussel bed to document the magnitude of the vertical stress gradient.

Field surveys of gonadal tissue color. At each of the 4 sites, mussels *Mytilus californianus* 5 to 7 cm in length were collected. This species reaches reproductive maturity at a size of ~2.5 cm (Suchanek 1981), and therefore all of these individuals were well above the size of reproductive maturity. Mussels were collected from the high (~2 m above mean lower low water [MLLW]) and low (~1 m above MLLW) edges of the mussel bed (15 individuals from each edge) and dissected in the field. Gonadal tissue color was scored on a scale of 1 to 3, with 1 as white, 2 as peach, and 3 as orange (Fig. 2), and sex was assessed in the field based on tissue color (white as male, peach as unknown, orange as female). Gonadal tissue was subsequently removed and placed into 10% formalin in seawater for histological processing to confirm sex.

Histological confirmation of sex. Gonadal tissues were dehydrated, embedded in paraffin wax, sliced to 7 µm thickness, and stained with hematoxylin and eosin according to Luna (1968). Slides were examined under a compound microscope, and sex was identified by the presence of either male or female post-gonial gametes.

Fig. 2. *Mytilus californianus.* Representative colors of mussel gonadal tissue for field color scoring system

Statistical analyses. Histological identification of sex was used to quantify the percentage of mussels that would have been sexed incorrectly based on the field color system. Males with color scores >2 would have been incorrectly classified as a female, and females with color scores <2 would have been incorrectly identified as a male. Males or females with color scores = 2 would have been classified as 'unknown' in the field. Data were arcsine–square root transformed prior to analyses to improve normality. Two analyses of variance (ANOVA) were performed across pooled time points, testing the effects of site, edge, sex, and the edge × sex interaction on the percentage of mussels that would be inaccurately sexed based on color: one ANOVA excluded the 'unknown' data and the other ANOVA included the 'unknown' data. Categories were compared with Tukey-Kramer honestly significant difference (HSD) tests based on least-square means estimated from main effects or interaction terms at $p < 0.05$ (Quinn & Keough 2002).

Survey data were analyzed with 4-factor ANOVA using JMP 6.0 (SAS Institute, Inc.) statistical software. Month, edge, site, sex, and all interactions were examined as explanatory variables, and color was the response variable. Residual and normal probability plots were examined for the presence of outliers and for normality. Color data were ln-transformed to meet assumptions of normality and independent error terms for all analyses. Categories were compared with Tukey-Kramer HSD tests based on least-square means estimated from main effects or interaction terms at $p < 0.05$ (Quinn & Keough 2002). An exploratory 4-factor ANOVA was then performed to determine differences between capes by testing the effects of month, edge, cape, sex, and all interactions on color.

RESULTS

Chlorophyll *a* and temperature

Chl *a* values were typically lower at the Cape Foulweather sites (FC and BB) than at the Cape Perpetua sites (YB and SH), and there was a pulse of high phytoplankton in July at all sites (Fig. 3a).

Monthly average high temperature (calculated from daily highs of temperature loggers in the middle of the mussel bed) was consistent between May and July and decreased slightly in August and September (Fig. 3b). Temperature varied among the 4 sites, with BB and SH being hotter on average than FC and YB. This could

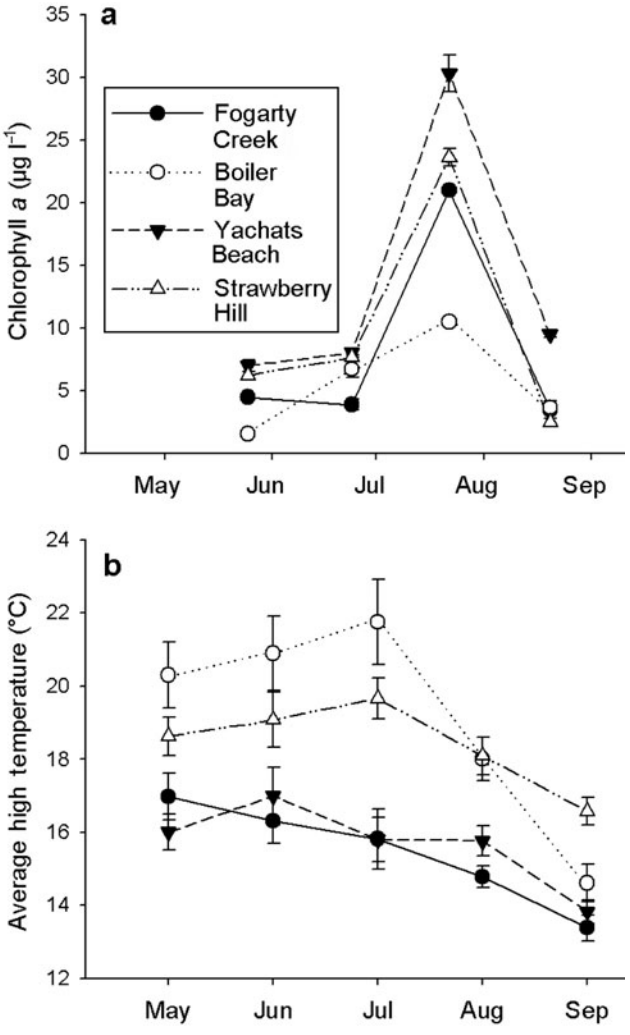

Fig. 3. Chlorophyll *a* (chl *a*) and temperature measurements at all 4 field sites from May to September 2004. Circles: Cape Foulweather sites; triangles: Cape Perpetua sites. (a) Monthly chl *a* measurements. Error bars = standard error of replicate bottle samples. (b) Monthly average high temperature based on daily high temperatures. Error bars = standard error of daily highs

either be due to true between-site differences or could instead be the result of microhabitat temperature differences due to placement of loggers (e.g. Helmuth & Hofmann 2001), because there was only 1 logger site^{-1}. For the present study, we will focus on temperature trends across all sites, rather than at any individual sites.

Within the vertical gradient at SH, monthly average temperatures (from daily averages) and monthly average high temperatures (from daily maxima) were consistently higher in the middle of the mussel bed than at the lower edge (Fig. 4), showing that thermal stress increases with tidal height.

Accuracy of using tissue color to identify sex of mussels

When 'unknown' (color score = 2) data were excluded, mussels *Mytilus californianus* at the high edge of the mussel bed were inaccurately sexed based on color more frequently (37.2 ± 9.1%) than mussels at the low edge (10.6 ± 3.1%; $F_{1,9}$ = 22.82, p = 0.001). More males (33.6 ± 10.3%) than females (14.2 ± 3.0%) were incorrectly sexed based on color ($F_{1,9}$ = 7.00, p = 0.03). High-edge males were sexed incorrectly 60.6% (±1.4%) of the time (Fig. 5), which was much more often ($F_{1,9}$ = 25.22, p = 0.0007) than low-edge males (6.5 ± 3.1%), low-edge females (14.8 ± 4.8%), and high-edge females (13.7 ± 4.5%). There were no differences between sites in the ability to correctly assign sex to mussels based on color ($F_{3,9}$ = 0.50, p = 0.69).

If the percentage classified as 'unknown' was added to the percentage scored incorrectly, the same differences resulted, except that the percentages misclassified increased (Fig. 5). Mussels at the high edge of the mussel bed were inaccurately sexed based on color more frequently (62.1 ± 9.5%) than mussels at the low edge (35.8 ± 4.4%; $F_{1,9}$ = 26.55, p = 0.0006). More males (64.7 ± 8.2%) than females (33.2 ± 4.5%) were incorrectly sexed based on color ($F_{1,9}$ = 37.60, p = 0.0002). High-edge males were misidentified more frequently (86.0 ± 3.8%; $F_{1,9}$ = 10.12, p = 0.01) than low-edge males (43.5 ± 1.7%), high-edge females (38.3 ± 5.6%), and low-edge females (28.1 ± 6.9%). There

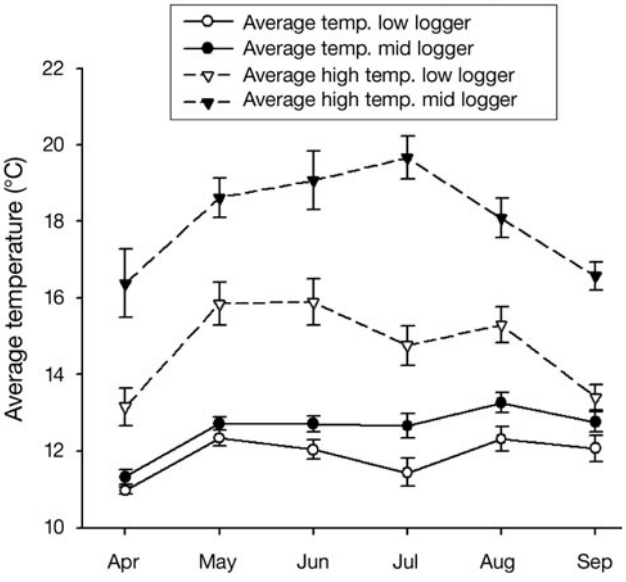

Fig. 4. Temperature measurements from Strawberry Hill temperature loggers between April and September 2004. Average monthly temperatures and average monthly high temperatures calculated from daily recordings of low- and mid-zone loggers. Error bars are standard error of daily average or daily high temperatures

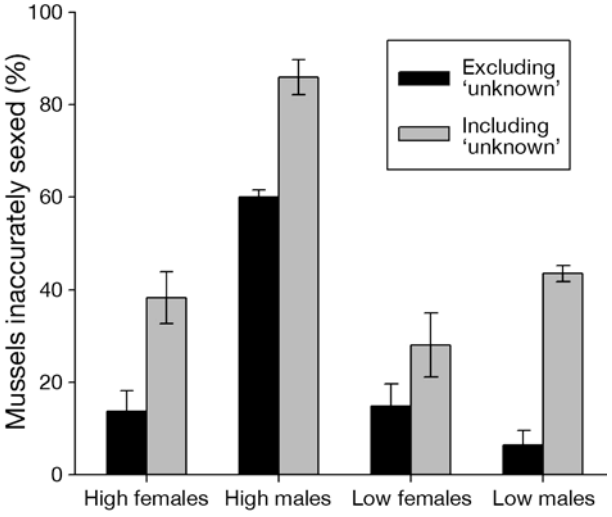

Fig. 5. *Mytilus californianus.* Percentage of mussels inaccurately sexed based on tissue color. Black bars: percent incorrectly scored not including 'unknown' category; grey bars: percent incorrectly scored including 'unknown' category. Error bars = standard error of 4 sites

were no differences between sites in the ability to correctly assign sex to mussels based on color ($F_{3,9}$ = 0.77, p = 0.54).

Patterns of gonadal tissue coloration

Color was more orange overall in the high-edge mussels (2.45 ± 0.03 on the 1 to 3 color index scale) than the low-edge mussels (2.11 ± 0.04; p < 0.0001; Fig. 6; see Table 1 for complete results of ANOVA). Females had higher overall levels of coloration (2.50 ± 0.04) than males (2.03 ± 0.04; p < 0.0001). However, there were no differences in color between high-edge males and females, which were all more orange (means from 2.44 to 2.53) than low-edge males (1.63 ± 0.04; p < 0.0001). BB had the most orange coloration (2.39 ± 0.05), and FC had the least (2.17 ± 0.06; p = 0.0004).

Overall, color decreased through time, as the mussels had higher levels of coloration in May and June (means from 2.54 to 2.70) than in July through September (means from 2.01 to 2.08; p < 0.0001). This is likely due to spawning activity and resulting loss of gametes from gonadal tissue throughout the summer (Petes et al. in press). In addition, color varied with time across the sites (p < 0.0001), with the highest level of orange coloration at YB in June (2.90 ± 0.06) and the lowest levels at FC in August (1.58 ± 0.08) and September (1.72 ± 0.11). Color varied with sex over time (p < 0.0001), as females in May and June had the highest levels of orange coloration (means from 2.97 to 2.98), and males in July through September had the lowest levels (means from 1.88 to 1.92). A relationship between sex, edge, and month on color (p = 0.007) revealed that females from both edges had more orange coloration earlier in the summer (means from 2.94 to 3.00) than low-edge males later in the summer (means from 1.38 to 1.55). A relationship between site, edge, and month on color (p = 0.002) showed that the highest orange coloration was found in mussels in the high edge at SH and FC in May and at YB and SH in June (means from 2.87 to 3.00), and the lowest was at the low edge at FC in August and September (means for both months = 1.33). FC, SH, and YB females in both May and June had the most orange coloration (means = 3.00), whereas FC males in August had the least (1.21 ± 0.07; p = 0.001).

The ANOVA performed with cape rather than site as an explanatory variable elucidated several of the com-

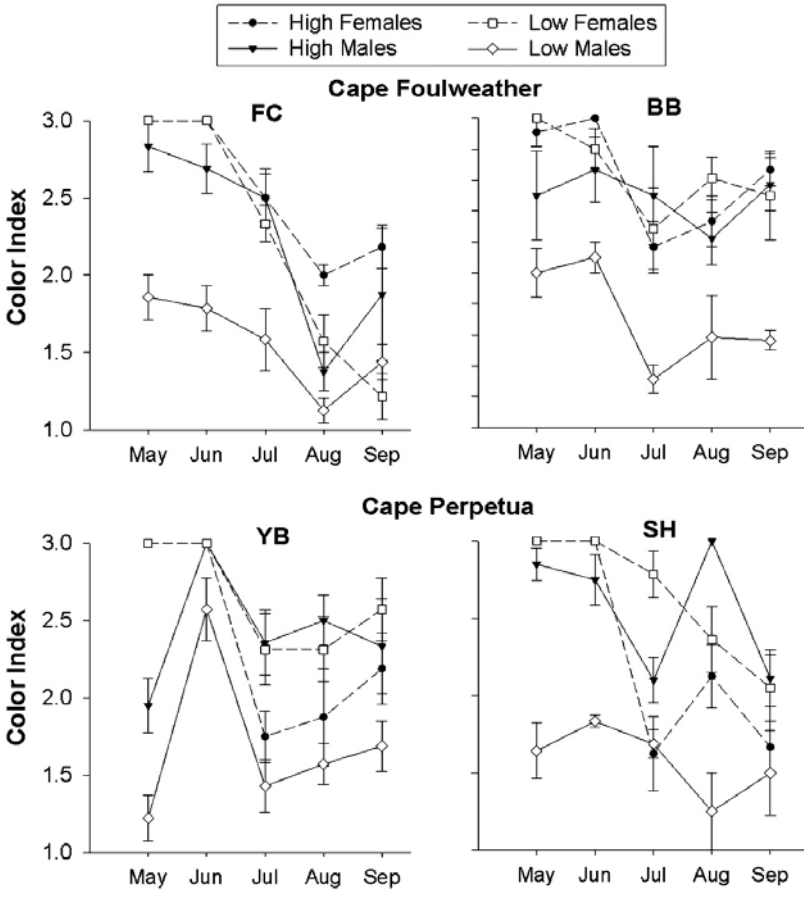

Fig. 6. *Mytilus californianus.* Average color index (1: white; 2: peach; 3: orange) of mussels collected from 4 field sites between May and September 2004. For site abbreviations see Fig. 1. Error bars = standard error

plex differences described above (see Table 2 for complete ANOVA results). Mussels at Capes Perpetua and Foulweather in May and June had the most orange coloration (means from 2.38 to 2.74), and mussels at Cape Foulweather in August and September had the least (means from 1.91 to 1.99; p = 0.005). Also, females at both capes in May and June (prior to spawning) were the most orange (means from 2.94 to 3.00), and males at Cape Perpetua in May and at Cape Foulweather in August were the least orange (means from 1.63 to 1.96; p = 0.008).

Table 1. *Mytilus californianus.* Results of 4-factor ANOVA examining effects of month, site, edge, sex, and all possible interactions on color (ln-transformed). All values with p < 0.05 are in boldface

Parameter	df	SS	*F*	p
Month	4	11.37	51.66	**<0.0001**
Site	3	1.03	6.22	**0.0004**
Edge	1	4.92	89.37	**<0.0001**
Sex	1	6.89	125.22	**<0.0001**
Edge × Site	3	0.38	2.34	0.07
Sex × Site	3	0.07	0.43	0.73
Sex × Edge	1	6.44	117.06	**<0.0001**
Edge × Site × Sex	3	1.19	7.20	**<0.0001**
Month × Site	12	4.70	7.12	**<0.0001**
Month × Edge	4	0.18	0.83	0.51
Month × Sex	4	1.37	6.24	**<0.0001**
Month × Sex × Edge	4	0.79	3.61	**0.007**
Month × Site × Edge	12	1.74	2.63	**0.002**
Month × Sex × Site	12	1.81	2.73	**0.001**
Month × Sex × Site × Edge	12	1.22	1.85	**0.04**
Error	509	28.01		
Total	588	73.12		**<0.0001**

Table 2. *Mytilus californianus.* Results of 4-factor ANOVA examining effects of month, cape, edge, sex, and all possible interactions on color (ln-transformed). All values with p < 0.05 are in boldface

Parameter	df	SS	*F*	p
Month	4	10.97	41.95	**<0.0001**
Cape	1	0.00	0.00	0.97
Edge	1	5.45	83.42	**<0.0001**
Sex	1	6.95	106.33	**<0.0001**
Edge × Cape	1	0.35	5.33	**0.02**
Sex × Cape	1	0.01	0.20	0.66
Sex × Edge	1	7.17	109.73	**<0.0001**
Edge × Cape × Sex	1	0.46	7.01	**0.008**
Month × Cape	4	0.98	3.74	**0.005**
Month × Edge	4	0.21	0.80	0.52
Month × Sex	4	1.61	6.16	**<0.0001**
Month × Sex × Edge	4	0.83	3.16	**0.01**
Month × Cape × Edge	4	1.01	3.86	**0.004**
Month × Sex × Cape	4	0.87	3.34	**0.01**
Month × Sex × Cape × Edge	4	0.18	0.68	0.61
Error	549	35.89		
Total	588	73.12		**<0.0001**

DISCUSSION

The present study documented that visual assessment of gonadal tissue color is not an accurate method for classification of sex in mussels and that this pattern varies with environmental stress. Male mussels *Mytilus californianus* showed differences in tissue coloration in relation to the vertical stress gradient in the intertidal zone, with most males having white gonadal tissue at the low edge of the mussel bed and orange gonadal tissue at the high edge.

Gonadal tissue color did not differ with food availability. As shown previously (Menge et al. 1997, 2004, Leslie et al. 2005), phytoplankton (chl *a*) concentrations were higher at the Cape Perpetua sites than at the Cape Foulweather sites. However, gonadal tissue color did not vary consistently among sites or between capes. Perhaps mussels in this environment are not food limited, as the central Oregon coast has a high phytoplankton abundance due to intermittent upwelling, and therefore they do not show a strong temporal color response to pulses in food availability.

Environmental stress (low-edge vs. high-edge environment) strongly influenced patterns of gonadal tissue coloration. High-edge mussels had a higher incidence of orange coloration than mussels from the low edge of the mussel bed. The most striking and consistent difference was found in gonadal pigmentation between low-edge and high-edge males. High-edge males had just as much orange coloration in their gonadal tissue as females. Well over half of the males from the high edge would have been scored incorrectly based on tissue color, indicating that this is an inaccurate method for assigning sex to *Mytilus californianus*, particularly at the high edge of the mussel bed, where the pattern of high orange coloration is most distinct. Using visual classification based on tissue color would have resulted in the incorrect interpretation that sex ratios are skewed, with a majority of mussels at the high edge being female. Based on these results, visual identification of sex should be examined for accuracy in other bivalve species, and the ability to correctly identify sex based on gonadal tissue color should be validated with histology prior to use in the field.

Orange coloration in mussels is due to the presence of carotenoid pigments (Campbell 1969, 1970, Petes et al. in press), and it appears that male mussels at the high edge of the mussel bed accumulate high concentrations of carotenoids into their gonadal tissues (Fig. 7; see also Petes et al. in press). This phenomenon has interesting implications with regards to the relationship between carotenoid pigments and the resistance to potential oxidative stress. Reactive oxygen species are generated in intertidal organisms as a conse-

Fig. 7. *Mytilus californianus*. Photograph of male mussel from the high edge of the mussel bed exhibiting bright orange gonadal tissue. Photograph courtesy of J. Lubchenco

quence of thermal stress experienced during aerial exposure at low tide (e.g. Abele et al. 1998), and these oxygen radicals can be extremely damaging to DNA, lipids, and proteins (Di Mascio et al. 1991). Carotenoid pigments are known for their antioxidant properties, because they can bind to singlet oxygen radicals and convert them to less-damaging hydrogen peroxide (Miki 1991). It is therefore possible that carotenoid pigments in high-edge male mussels protect gametes from oxidative damage.

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