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Season- and sex-related variation in mucin secretions of the striped Venus clam, *Chamelea gallina* (Linnaeus, 1758) (Bivalvia: Veneridae)

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Abstract
An *in situ* analysis of mucin secretions along the annual cycle was performed on the striped Venus clam, *Chamelea gallina*. Individuals of both sexes from an exploited stock of Margherita di Savoia (Southern Adriatic Sea, Central Mediterranean Sea) were made in June 2020 and 2021, and in January 2021 and 2022, representative of summer and winter seasons, respectively. Tissues from the foot and the gills were analyzed for histochemical and lectin-histochemical analyses. Staining with Periodic Acid-Schiff, Alcian Blue pH 2.5 and High-Iron-Diamine indicated that mucins from both the foot and the gills were acidic, mostly sulfated. Lectin-binding analyses with PNA, SBA, WGA, LTA, UEA-I, AAA, SNA, MAA-II and ConA indicated the presence of N-acetyl-glycosaminylated, mannosylated and fucosylated residuals in the saccharidic chains. In the gills, the amount of acidic and glycosaminylated residuals was higher in summer in both sexes, whereas fucosylation was similar along the sampling period and mannosylation was never observed. In the foot, both surface epithelial and subepidermal secreting cells increased sulfation in winter in males, but not in females. Glycosaminylation and fucosylation were observed only in the surface cells of males. Mannosylated residuals were observed in all the foot cell types in both sexes. It is hypothesized that the observed qualitative and quantitative variations in mucin secretion is linked to the reproductive cycle.

Highlights

- The mucocytes present in the gill filaments of the clam produce an acid mucus, with sulfate residues and N-acetylglucosamine and fucose chains.
- In the foot, the subepithelial mucocytes produce an acidic, sulfate secretion with mannosylated residuals.
- The foot epithelium shows an acidic, sulfated mucus composition with N-acetylglucosamine, fucose and mannose chains.
- The results show that males have greater variability in mucus composition.
- The observed variation could be related to different energy allocation between sexes in the development of gametes.

Keywords: Chamelea gallina, *glycoconjugates*, *gill*, *foot*, *seasonal cycle*

Introduction

Mucins are highly glycosylated proteins representing major components of the mucus secreted by the epithelia of mollusks. They are involved in several functions, such as filter feeding, digestion, breathing and cleaning of the mantle cavity, protection against predators and pathogens, adhesion and/or penetration in the substrate, osmoregulation, biomineralization, intra- or interspecific signaling by luminescence (Bolognani-Fantin et al. 1969; Prezant 1990; Jørgensen 1996; Davies & Hawkins 1998; Espinosa et al. 2009). Moreover, the mucins dispersed in the environment in pseudofaeces contribute to the particulate organic matter in coastal waters (Davies & Hawkins 1998). In

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the mucin structure, the oligosaccharidic chains are usually attached to the protein backbone by an α-O-glycosidic linkage to a serine or a threonine. Variations in glycosylation and/or carbohydrate composition of the saccharidic chains affect the viscoelastic, lubricating and hydration properties and thus the functions of mucus (Bansil & Turner 2018). Despite their importance, studies about mucin compositions, distribution and variation in molluscan secretions are still scarce. Classical histochemical studies indicate only that the oligosaccharidic chains in the mucus can be neutral or acidic, due to the presence of carboxylated or sulfated residuals (e.g., Bolognani-Fantin et al. 1969; Ottaviani et al. 1990; Albanese & Calabrò 1996; Eble 2001; Calabrò et al. 2005; Petraccioli et al. 2013). *In situ* studies about the composition of the oligosaccharidic chains are scarcer and indicate the presence of mannosylated, glycosaminylated, galactosyl/galactosaminylated and fucosylated residuals, whereas the presence of sialic acid is debated (e.g., Ahn et al. 1988; Ottaviani et al. 1990; Albanese & Calabrò 1996; Robledo et al. 1997; Calabrò et al. 2005; Bravo Portela et al. 2012; Petraccioli et al. 2013). Glycosylation has been observed to vary from one secreting cell type to another, distributed in different anatomical districts of the mollusks such as the mantle, the foot, the gills, or the digestive gland (e.g., Bolognani-Fantin et al. 1969; Beninger & St-Jean 1997; Eble 2001; Calabrò et al. 2005; Nogarol et al. 2012; Bilgin & Uluturhan-Suzer 2017). Besides, in some classes of this phylum such as gastropods and cephalopods, the composition of mucins has been observed to change in a same cell type in relation to physical variables (hydrodynamism, salinity, temperature, humidity) or animal activities (reproduction, growth, locomotion, reproduction and feeding) (e.g., Davies et al. 1990; Petraccioli et al. 2013; Accogli et al. 2017). Moreover, qualitative and quantitative variation of mucins have also been demonstrated to be affected by pollutants, suggesting that their variations can be used as a biomarker (e.g., David & Fontanetti 2009; Cutuli et al. 2021; Guglielmi et al. 2022). Being filter-feeding organisms capable of consuming pollutants from the environment, the bivalves are widely used in the monitoring of water quality (Goldberg 1975; Kádár et al. 2001; March et al. 2007; Helmholz et al. 2016) and they are regarded as a model for human health (Fernández Robledo et al. 2019).

With all the previous as reference, we performed a qualitative and quantitative *in situ* analysis of mucin secretion along the annual cycle of a commercial mollusk bivalve, the striped Venus clam, *Chamelea gallina* (L., 1758). This species is common in well-sorted sand biocenosis (Pérès 1967; Montefalcone et al. 2021) along the shallow coastal areas of the western Atlantic, from Norway to North Africa, as well as along the Mediterranean and the Black Sea coasts (Orban et al. 2007; Begun et al. 2022). Therefore, knowledge about the mucin composition in this species and its variation in relation to sex or season could represent a reference point for further studies in which biomarkers from this species will be used in responses to environmental stresses.

Material and methods

Study animals

Commercial-sized individuals of *C. gallina* were sampled by a commercial clam dredger in an exploited fishing ground of Margherita di Savoia (BT) in Southern Adriatic Sea (Cascione et al. 2022).

Four samplings were carried out in June 2020, January, and June 2021 and January 2022, representative of summer and winter conditions, in which the gonads are in different stages of development (Erkan & Sousa 2002; Dalgiç et al. 2009; Erkan 2009; Delgado et al. 2013). Animals collected were immediately transported to the University of Bari, and 10 specimens for each sampling were randomly selected (shell length, mean ± SD: 27.5 ± 2.8 mm; weight with shell 6.1 ± 2.1), sedated with magnesium chloride (Ross & Ross 2009) and fixed in 4% seawater buffered formalin for 24 h. Subsequently, the individuals were washed in freshwater and dehydrated in a graded series of ethanol for paraffin embedding. Sections, 5 μm-thick, were cut and collected on slides. A preliminary stain of sections with Toluidine blue was made to observe the general morphology of animals, sex determination and stage of gametogenesis in the gonads according to Dalgiç et al. (2009) and Delgado et al. (2013). Since different gametogenic stages were seen simultaneously in the same individual, their reproductive status was defined based on the most representative condition (Delgado et al. 2013).

Samples of clams were selected for mucin analyses in the gill and foot epithelium

Histological and histochemical staining

General detection of carbohydrates with 1,2 vicinal diols was performed with Periodic-Acid Schiff (PAS: McManus 1948). Since the mucins are rich in O-linked oligosaccharidic chains, their presence was demonstrated by removing them with β-elimination (Ono et al. 1983) followed by PAS staining. The sections were incubated with 0.2 M KOH in a solution of dimethylsulphoxide, water and ethanol (50:40:10) for 1 h at 45°C, then neutralized with 10 mM HCl and washed in phosphate-buffered saline (PBS) with pH 7.4 prior to making the PAS staining (refer to Mastrodonato et al. 2009, for further details). If PAS-positivity is due to mucins, its reduction or disappearance is expected after β-elimination, as observed by Petraccioli et al. (2013) in the foot of the limpet *Patella caerulea*. A diastase-PAS (Lillie & Greco 1947) was also performed to detect glycogen, as in Mentino et al. (2017). Before PAS-staining, the sections were incubated with 0.8% diastase in PBS in a humid chamber at 37°C overnight. In this case, the reduction of PAS positivity is explained by the removal of glycogen. Alcian Blue pH 2.5 (AB2.5) was used to detect acidic carbohydrates (Steedman 1950), while High-Iron-Diamine (HID: Spicer et al. 1978) was performed to detect sulfated carbohydrates. In all essays, counterstain with hematoxylin was performed to evidence the nuclei. Details about these histochemical techniques can be found in Scillitani et al. (2011). All the chemicals cited in this section were from Sigma-Aldrich (St. Louis, MO, USA).

Lectin binding experiments

Binding of nine fluorescein-isothiocyanate (FITC) conjugated lectins (PNA, SBA, WGA, LTA, UEA-I, AAA, SNA, MAA II, ConA) was performed to characterize the structure and distribution of the oligosaccharidic chains of the mucins. Lectins were selected among those detecting the most common oligosaccharidic chains in mollusks (e.g., Ottaviani et al. 1990; Calabrò et al. 2005; Petraccioli et al. 2013; Accogli et al. 2017). Details about lectin common names and sugar specificities are shown in Table I. Binding was obtained by incubating the rehydrated sections in the dark for 1 hour at room temperature with lectin diluted in 10 mM Hepes pH 7.5, or in 100 mM PBS pH 7.4 (buffer details and working concentrations for each lectin are in Table I). Sections were then rinsed in the same buffer and mounted with Fluoromount™ Aqueous Mounting Medium F4680 for observation. For PNA and SBA lectins, binding experiments were also performed after desulfation (as reported in Scillitani & Mentino 2015). The specificity of the lectins was tested by substituting the lectin solution with the buffer alone or preincubating the sections with the corresponding hapten sugar inhibitor (sugar types and concentration are in Table I). Specificity was also tested by binding to samples from other sources presenting mucins that were previously demonstrated to be labelled by lectins tested in this paper (Petraccioli et al. 2013; Accogli et al. 2017). The chemicals cited were from Sigma-Aldrich Inc. (St. Louis, MO, USA); lectins were

Table I. FITC-Lectins used with related binding specificity, diluting buffer, working dilution and inhibiting sugars used in negative control experiments.

Lectin	Origin	Binding sites	Diluting buffer	Dilution mg/ml	Inhibitory sugar concentration
PNA	*Arachis hypogaea*	Galβ1,3GalNAc	Hepes	10	0.2 M Gal
SBA	*Glycine max*	GalNAc	Hepes	20	0.2 M GalNAc
WGA	*Triticum vulgaris*	(GlcNAcβ1,4)n	Hepes	20	0.5 M GlcNAc
LTA	*Tetragonolobus purpureus*	L-Fucα1,6GlcNAc; L-Fucα1,2Galβ1,4[LFuc1,3] GlcNAcβ1,6 R	PBS	20	0.2 M L-Fuc
UEA-I	*Ulex europaeus*	Fucα1,2	Hepes	10	0.2 M L-Fuc
AAA	*Aleuria aurantia*	Fucα1,6GlcNAc-βNAsn; Fucα1,3, Fucα1,4	Hepes	10	0.2 M L-Fuc
SNA	*Sambucus nigra*	Neu5Acα2,6 Gal/GalNAc	PBS	20	0.2 M Neu5Ac
MAA-II	*Maackia amurensis*	Neu5Acα2,3Galβ1,4GlcNAc	PBS	20	0.2 M Neu5Ac
ConA	*Canavalia ensiformis*	D-Man, D-Glc	PBS	20	0.1 M MαM

from Vector Laboratories (Burlingame, CA, USA) except for AAA and MAA-II from US Biological (Salem, MA, USA).

Image and quantitative analysis

Brightfield histological images were acquired using a Nikon Eclipse E600 microscope equipped with DA-Fi3 microscope-camera (Nikon Instruments SpA, Campi Bisenzio, FI, Italy), and acquisition software NIS-Elements D 5.30.00 64-bit. The same microscope equipped with an epifluorescence device under 495 nm light emission was used for the acquisition of the images documenting lectin-binding.

For each stain or lectin experiment, 10 images per individual were taken (five for the foot and five for the gills). In each image, 10 to 20 cells of each type having the best orientation were selected for the analyses.

For conventional histochemistry (PAS, AB pH 2.5, HID), the intensity of staining was estimated by computing optical density (OD) values. Colour deconvolution (Ruifrok & Johnston 2001) was used to isolate colour channels resulting from staining and to exclude the counterstaining and the background (Mastrodonato et al. 2020). Similarly, lectin-binding intensity was estimated by computing the corrected total cell fluorescence (CTFC) (McCloy et al. 2014). Computations for image analysis were obtained with the Fiji package by ImageJ (Rasband 2022) implemented with the colour deconvolution plug-in (Landini et al. 2021).

Mean OD or CTFC values from each staining, cell type and condition were compared by One-Way Analysis of Variance (ANOVA) followed by resampling through a 10.000-iteration bootstrap of the F-stat values (e.g., Carlucci et al. 2022). Differences between means by post-hoc pairwise comparisons were tested by Tukey's Honestly-Significant-Difference (THSD). Significance of probability was set at $p < 0.01$. Statistical computations were generated by the Real Statistics Resource Pack plugin for Excel (Release 4.3: Zaiontz 2020).

Results

The preliminary histological analysis identified 24 males and 16 females. Both sexes were in reproduction period in June (2020 and 2021), with mature gametes observed in the gonads (Figure 1(a,c)). There were several bands of spermatocytes in males (Figure 1(a)) and many large oocytes, most free from peduncles in females (Figure 1(c)), in some cases with partial release. By January (2021 and 2022), the specimens were in gametogenesis. In males many spermatocyte bands were already visible (Figure 1(b)), whereas females showed with small, pedunculated oocytes (Figure 1(d)).

Histological and histochemical staining

The gills of *C. gallina* present several filaments with a ciliated frontal region (Figure 1(e)). One main mucocyte is seen on each side of the filament (Figure 1(e)). In the foot, two secreting cell types are observed, arranged into groups, i.e., surface and subepidermal cells. Single surface cells are distributed among the ciliated cells in the apical surface epithelium, whereas the subepidermal cells are observed in the connective tissue underlying the surface epithelium and are clustered in pluricellular glands (Figure 1(f-h)).

PAS staining resulted always negative (Figure 1(e)) in the gills, whereas it was positive in both the surface and subepidermal foot cells (Figure 1(f)). PAS-positivity persisted after the diastase treatment, indicating that the stained glycans are not glycogen (Figure 1(g)). On the contrary, PAS-positivity greatly decreased after β-elimination (Figure 1(h)), suggesting that the saccharidic chains were removed because they are O-linked and thus confirming that PAS-stained glycans are mucins. PAS-positivity in the foot glands showed a significant variation among seasons (Figure 2), with a decrease observed in both sexes in January (ANOVA: surface cells, $F_{7,\ 1958} = 1461.465$, $p = 0.000$; subepidermal cells $F_{7,\ 2005} = 124.268$, $p = 0.000$).

AB pH 2.5 staining was positive in both the gills and the foot glands (Figures 3 and 4). In the gills, OD intensity in both sexes decreased significantly in January (ANOVA: $F_{7,\ 3977} = 1593{,}487$; $p = 0.000$). Similar to the gills, AB positivity in the foot cells (Figure 4) decreased significantly in both sexes in January (ANOVA: surface cells, $F_{7,\ 1967} = 156.062$, $p = 0.000$; subepidermal cells $F_{7,\ 1994} = 449.034$, $p = 0.000$).

HID stained the gill filament mucocytes in both sexes (Figure 5) and significant seasonal variation was found (ANOVA: $F_{7,\ 3983} = 2345{,}65$; $p = 0.000$), but patterns differed between sexes: in males, a higher intensity was observed in June (Figure 5(a,c)), whereas in females, it was observed

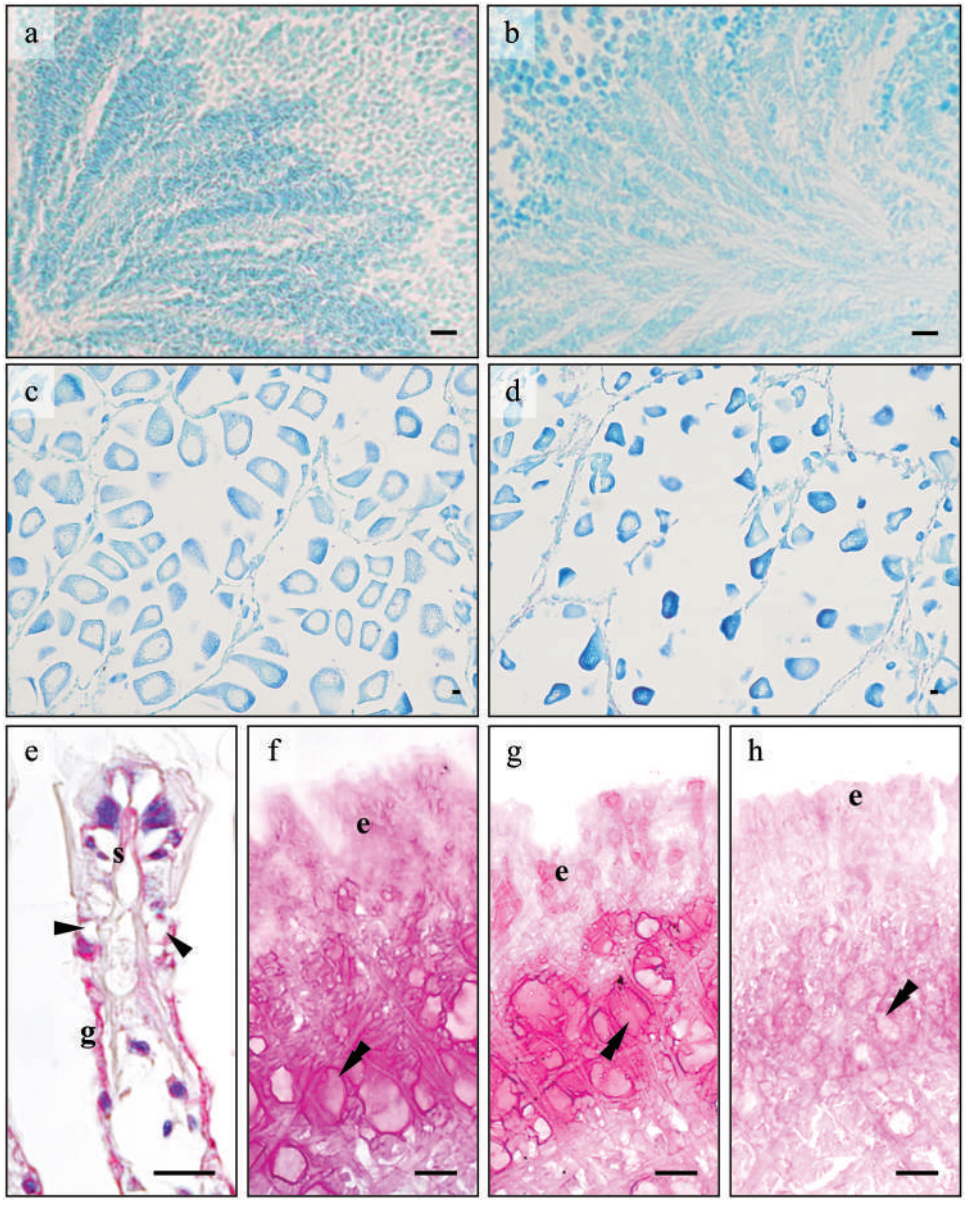

Figure 1. Tissues of the striped Venus clam, *Chamelea gallina* treated with histological and histochemical stains. (a-d) Gonads stained with Toluidine Blue. In the male gonad (a-b) little variation in the number of sperms is observed between January (e) and June (f), suggesting that spermatogenesis is already in an advanced state in the former period. In the female gonad (c-d) early ovogenetic stages are observed in January (g) in respect to June (h), where mature eggs ready to spawn are seen. (e) Gill epithelium, PAS-Hematoxylin. Mucocytes (arrowhead) on gill filaments are always PAS negative. Positivity is sometimes observed on the glycocalyx (g) and chitin skeleton (s). (f-h) Foot epithelium stained with PAS (f), diastase-PAS (dPAS) (g), and β-elimination-PAS (βPAS) (h) methods. PAS positivity does not vary with dPAS whereas is reduced with βPAS, indicating that positivity in the surface (e) and subepidermal cells (double arrowhead) is due to mucins with O-linked saccharidic chains and not to glycogen. Abbreviations: e, surface cells; g, glycocalyx; s, chitin skeleton of gill filament; Scale bars: 10 μm.

in January (Figure 5(f,h)). In the foot (Figure 6), significant seasonal variation was observed for both secreting cells (Figure 6(a-h)) in the males only (ANOVA: surface cells, $F_{7,\ 1987} = 728.254$, $p = 0.000$; subepidermal cells $F_{7,\ 1991} = 3203.789$, $p = 0.000$).

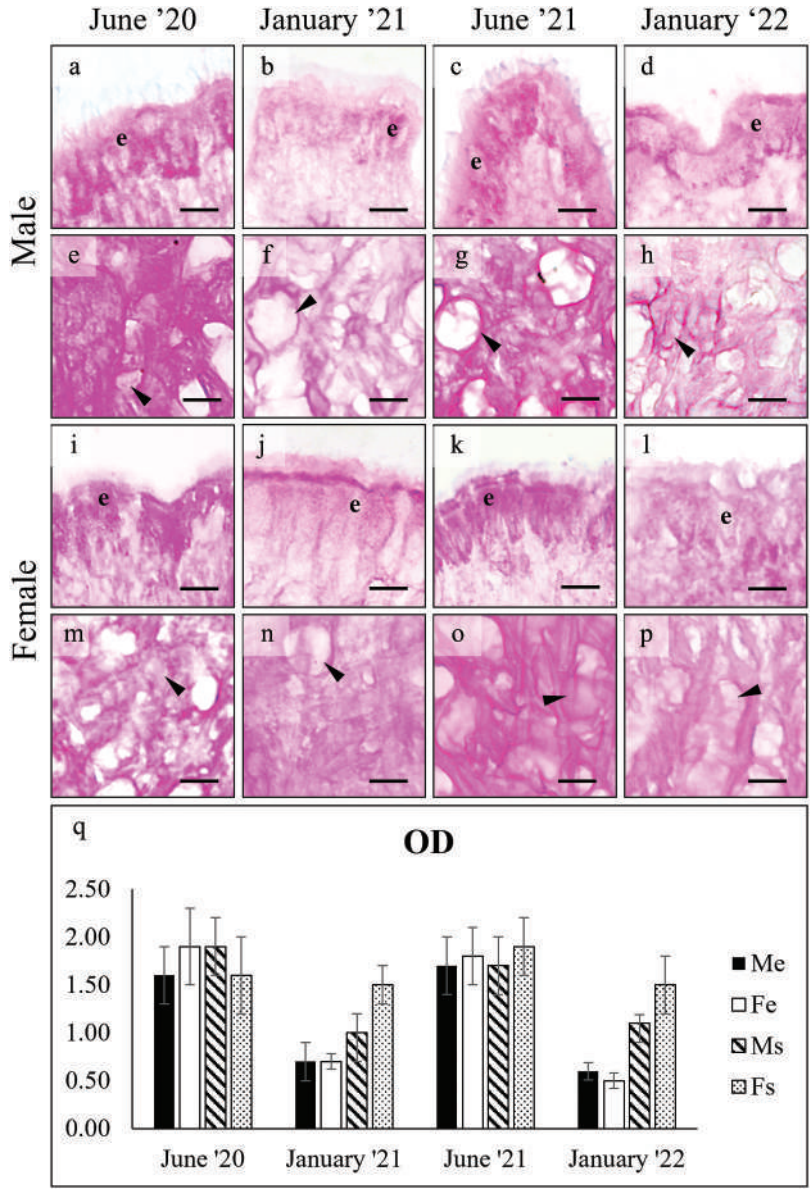

Figure 2. PAS staining of secretory cells in the foot of male (a-h), and female (i-p) *C. gallina*. For each sex, the top images (a-d for males; i-l for females) show the variation of secretion between summers (June) and winters (January) conditions in the surface cells (e), whereas the bottom images (e-h for males; m-p for females) shows the stain of subepidermal glands (arrowheads) in the same periods. (q) Plot of mean OD values for male surface cells (black), female surface cells (white), male subepidermal cells (striped), and female subepidermal cells (dotted). Summer values (June 20 and June 21) are significantly higher than the corresponding winter ones (January 21 and January 22), except for subepidermal cells in females (Fs). Abbreviations: Fe, surface cell in female; Fs, subepidermal cell in female; Me, surface cell in male; Ms, subepidermal cell in male. Scale bars: 10 μm.

Lectin binding experiments

Only three lectins (WGA, AAA and ConA) bound to the clam tissues.

WGA stained both the gills and the foot secreting cells with significant variation among seasons. In the gills (Figure 7) the mucocytes of both sexes stained more intensely in June than in January (ANOVA: $F_{7,\ 4003} = 1145.330$; $p = 0.000$). A second mucocyte was stained in a more apical position with respect to the main lateral one (Figure 7). In the foot, WGA stained intensely the surface cells and the glycocalyx of males (Figure 8(a-d)) ($F_{7,\ 1987} = 728.254$, $p = 0.000$) while those of females were significantly less intense (Figure 8(i-l)), without season variation. The subepidermal cells of females did not stain (Figure 8(e-h, m-p)).

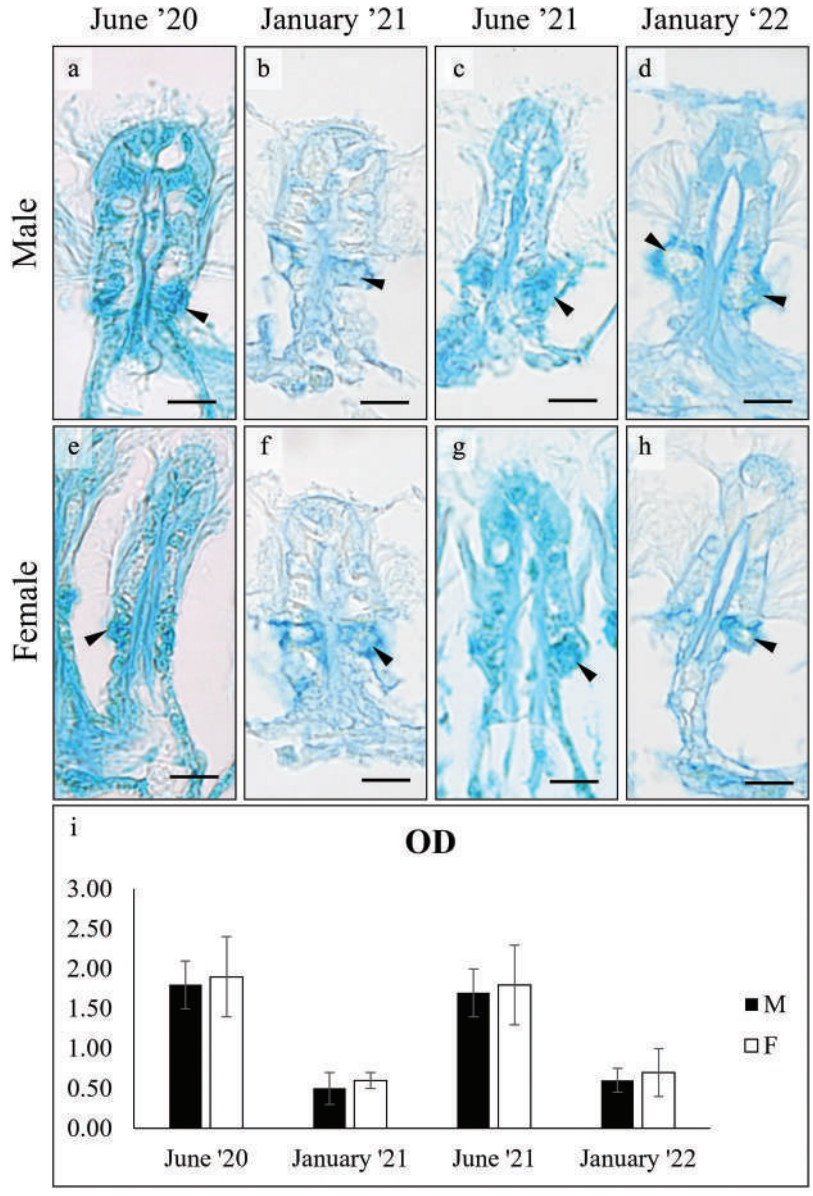

Figure 3. Alcian Blue pH 2.5 staining of mucocytes (arrowhead) on the gill filaments of male (a-d) and female (e-h) *C. gallina*. (i) Plot of mean OD values for gill mucocytes in males (black) and females (white) shows that summer values are significantly higher than the corresponding winter ones (January 21 and January 22). Abbreviations: F, female; M, male. Scale bars: 10 μm.

In the gills, the AAA stained intensely the mucocytes of males and females in each season (Figure 9). In the foot, the only cells stained by AAA were the surface ones in males (Figure 10(a-d)), with a significant increase in January ($F_{7,\ 2002}$ = 6265.066, p = 0.000). Glycocalyx was stained in both male and female surface cells (Figure 10 (a-d, i-l)).

ConA did not stain the gills, whereas both the secreting cells in the foot and the surface glycocalyx were bound intensely (Figure 11). In both sexes, a significant increase was observed in June with respect to January (Figure 11(a-d, i-l)), whereas the opposite was found in the subepidermal glands (Figure 11(e-h, m-p)) (ANOVA: surface cells, $F_{7,\ 1949}$ = 347.566, p = 0.000; subepidermal cells $F_{7,\ 2001}$ = 65.304, p = 0.000).

Discussion

The gill and foot epithelia of *C. gallina* are similar to those observed in other species of the same family Veneridae, such as *Mercenaria mercenaria* (Eble 2001) and *Ruditapes philippinarum* (Calabrò et al. 2005).

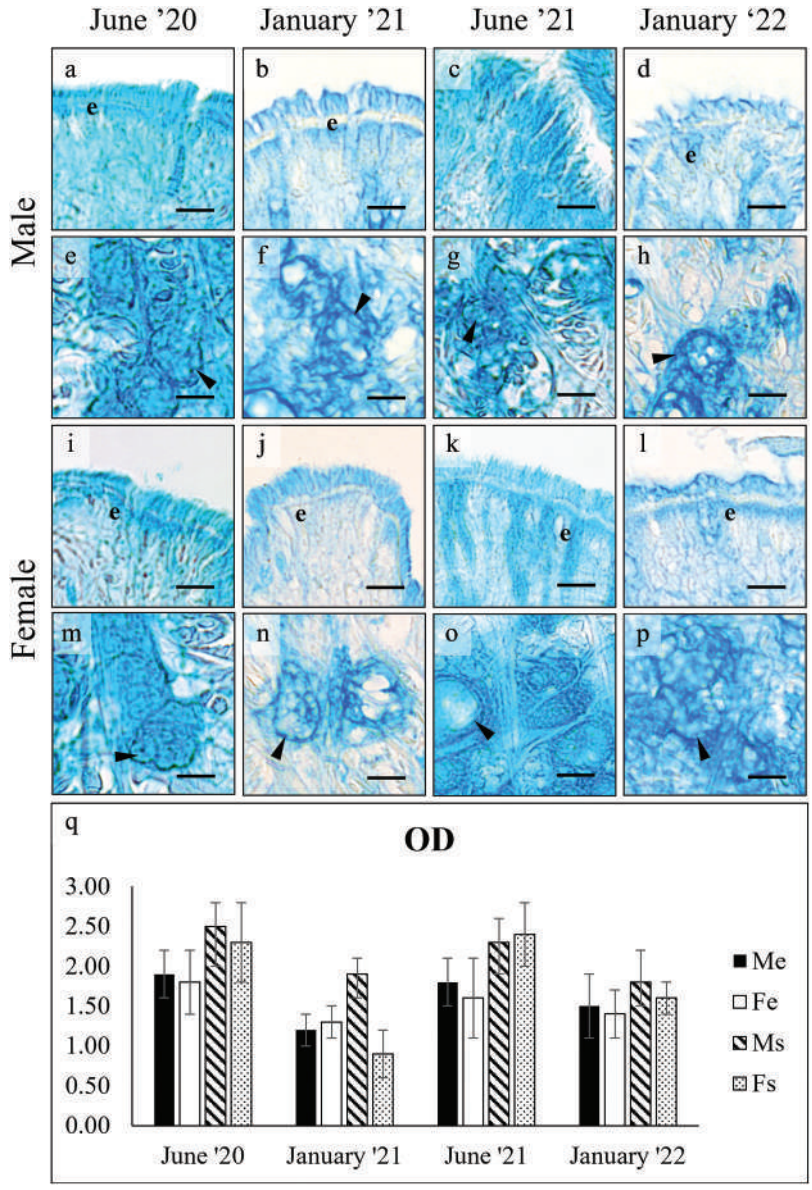

Figure 4. Alcian Blue pH 2.5 staining of secretory cells in the foot of male (a-h), and female (i-p) *C. gallina*. For each sex, the top images (a-d for males; i-l for females) show the variation of secretion between summers (June) and winters (January) conditions in the surface cells (e), whereas the bottom images (e-h for males; m-p for females) shows the stain of subepidermal glands (arrowheads) in the same periods. (q) Plot of mean OD values for male surface cells (black), female surface cells (white), male subepidermal cells (striped), and female subepidermal cells (dotted). Summer values (June 20 and June 21) are significantly higher than the corresponding winter ones (January 21 and January 22). Abbreviations: Fe, surface cell in female; Fs, subepidermal cell in female; Me, surface cell in male; Ms, subepidermal cell in male. Scale bars: 10 μm.

Positivity to AB pH 2.5 and HID indicates that the mucins secreted by this species from both the gills and the foot are acidic, with carboxylated and sulfated residuals. The latter prevail in the gills, where PAS positivity was not observed, suggesting the exclusive presence of acidic glycosaminoglycans (GAGs: e.g., Hooghwinkel & Smits 1957). As noted previously, acidic mucins are very common in mollusks and *C. gallina* confirms this trend.

As far as lectin binding is concerned, the mucins of *C. gallina* lack some residuals observed in other species, such as galactose and N-acetylgalactosamine, found in *Ruditapes philippinarum* (Calabrò et al. 2005) and *Codakia orbicularis* (Gourdine and Smith-Ravin 2002). In *C. gallina*, the sulfated residuals can interfere with lectin binding, in particular, PNA and SBA (e.g., Maupin et al. 2012); notwithstanding a preliminary desulfation treatment, the cited lectins

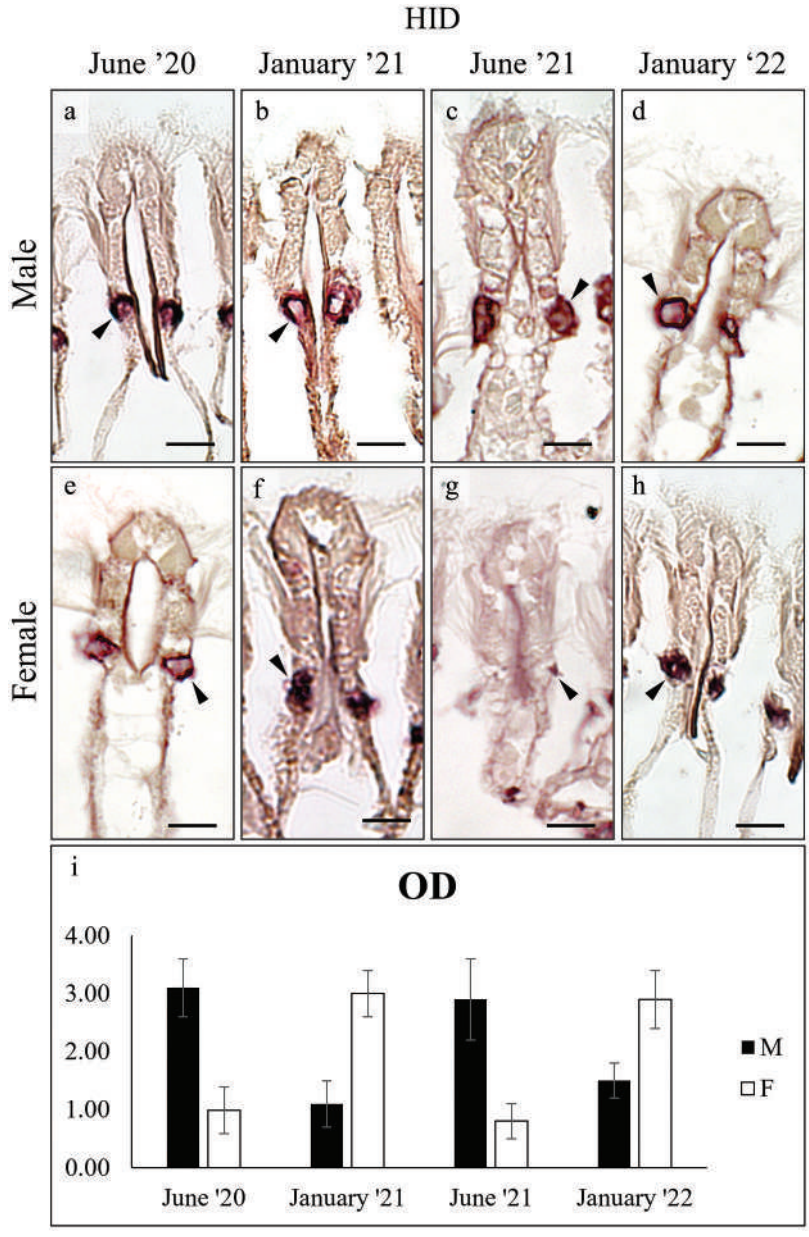

Figure 5. High Iron Diamine (HID) staining of mucocytes (arrowhead) on the gill filaments of male (a-d) and female (e-h) *C. gallina*. (i) Plot of mean OD values for gill mucocytes in males (black) and females (white) shows that summer values are significantly higher than the corresponding winter ones (January '21 and January '22) in males, whereas the opposite is seen in females. Abbreviations: F, female; M, male. Scale bars: 10 μm.

did not bind, indicating the absence of galactosyl/galactosaminyl residuals in the oligosaccharidic chains. Positivity to WGA can be explained by both the presence of N-acetylglucosamine and sialic acid (Gallagher et al. 1985). A comparison of our results from AB pH 2.5 staining with those from lectin binding suggests that in the mucins of *C. gallina* residuals of sialic acid are lacking. In particular, the subepidermal glands are positive to AB pH 2.5, but not to WGA and all the gland cell types were negative to the lectins specific to sialic acid (SNA and MAA II). Thus, we can conclude that positivity to WGA is due to glycosaminylated residuals only. This confirms the finding of Bolognani-Fantin et al. (1969) who did not find sialic acid in the foot and the mantle of a number of marine and freshwater bivalves.

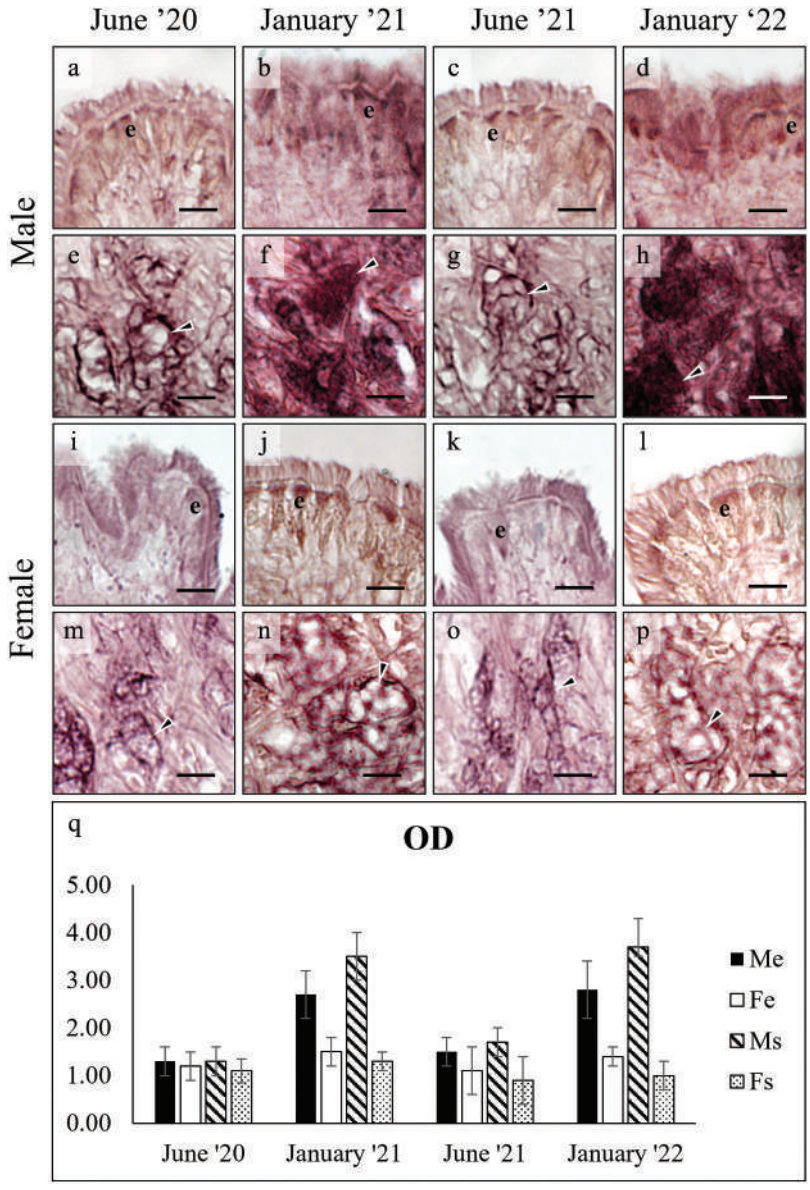

Figure 6. High Iron Diamine (HID) staining of secretory cells in the foot of male (a-h), and female (i-p) *C. gallina*. For each sex, the top images (a-d for males; i-l for females) show the variation of secretion between summers (June) and winters (January) conditions in the surface cells (e), whereas the bottom images (e-h for males; m-p for females) shows the stain of subepidermal glands (arrowheads) in the same periods. (q) Plot of mean OD values for male surface cells (black), female surface cells (white), male subepidermal cells (striped), and female subepidermal cells (dotted). Summer values (June 20 and June 21) in males are significantly lower than the corresponding winter ones (January 21 and January 22). In female moderate, non-significant variation is seen. Abbreviations: Fe, surface cell in female; Fs, subepidermal cell in female; Me, surface cell in male; Ms, subepidermal cell in male. Scale bars: 10 μm.

According to Ottaviani et al. (1990), in gastropods, sialic acid is substituted by N-acetylmuramic acid, and this could also be the case of *C. gallina*, since WGA can also link N-acetylmuramic acid residuals (Jarroll et al. 1989). On the contrary, sialic acid is reported by Ahn et al. (1988) in the mucous cells of the gills of the mussel *Mytilus edulis* and by Calabrò et al. (2005) in the foot and the mantle of the venerid *Ruditapes decussatus*.

Positivity with AAA and ConA binding indicates that in the mucins of *C. gallina* there are fucosylated and mannosylated residuals, respectively. Mannose apart, ConA can link to glucose residuals (Finne & Krusius 1982), but the negative results from

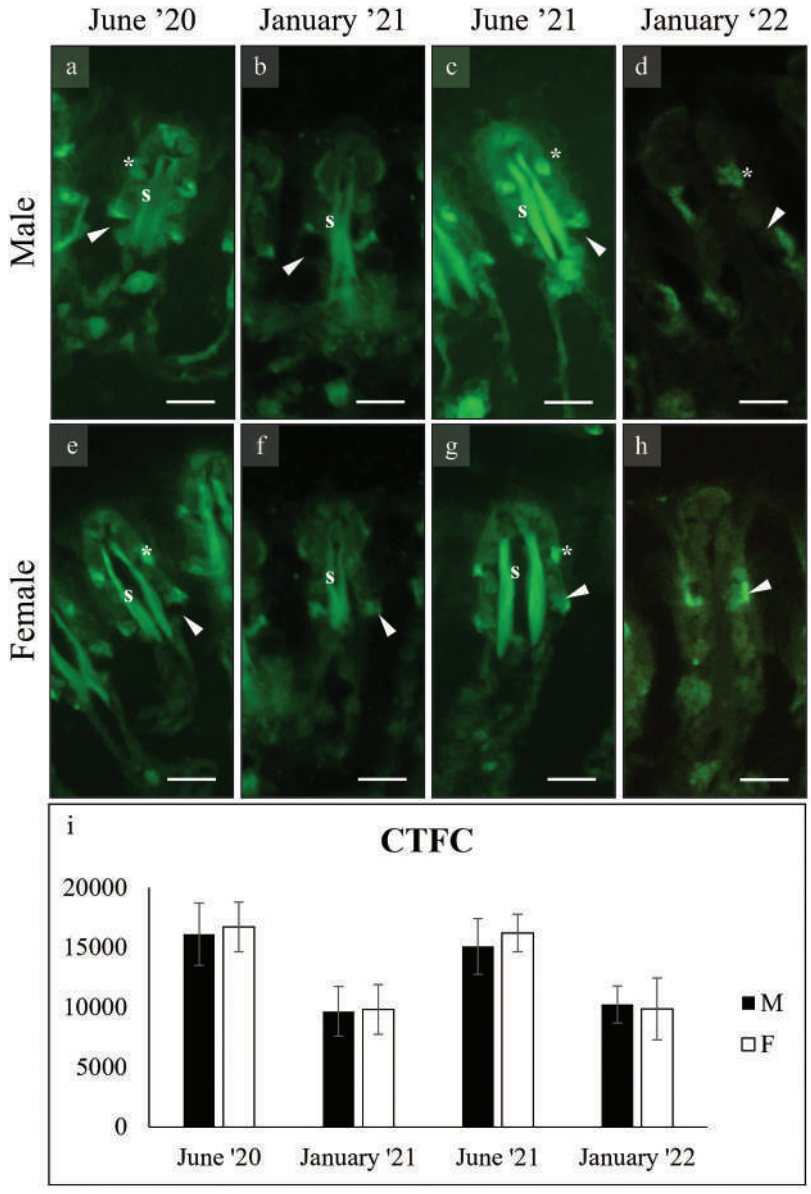

Figure 7. WGA binding of mucocytes (arrowhead) on the gill filaments of male (a-d) and female (e-h) *C. gallina*. WGA binds also to the chitin skeleton of filaments (s) and to a second type of mucocyte (asterisk) in the frontal area. (i) Plot of mean CTFC values for gill main mucocytes in males (black) and females (white) shows that summer values are significantly higher than the corresponding winter ones (January 21 and January 22). Abbreviations: F, female; M, male; s, chitin skeleton of gill filament; *, frontal mucocyte. Scale bars: 10 μm.

diastase-PAS experiments indicate the lack of glycogen, and thus large amounts of glucose, in the sampled tissues.

As far as the functional interpretation of the evidenced glycosidic residuals is concerned, sulfated and fucosylated chains could be involved in mucus viscosity and its defense properties (e.g., Denny 1983). Glucosaminylated residuals can provide attaching sites for sulfates or other carbohydrates (Menghi et al. 1991; Esko et al. 2009). A mannosylated core in mucins can have structural and regulative functions (Esmail & Manolson 2021).

The present study found extensive quantitative and qualitative variation of mucins related to cell specificity, life cycle and sex of *C. gallina*.

The gill glands present a lower diversity of residuals with respect to the foot showing oligosaccharidic chains with sulfated, glycosaminylated and

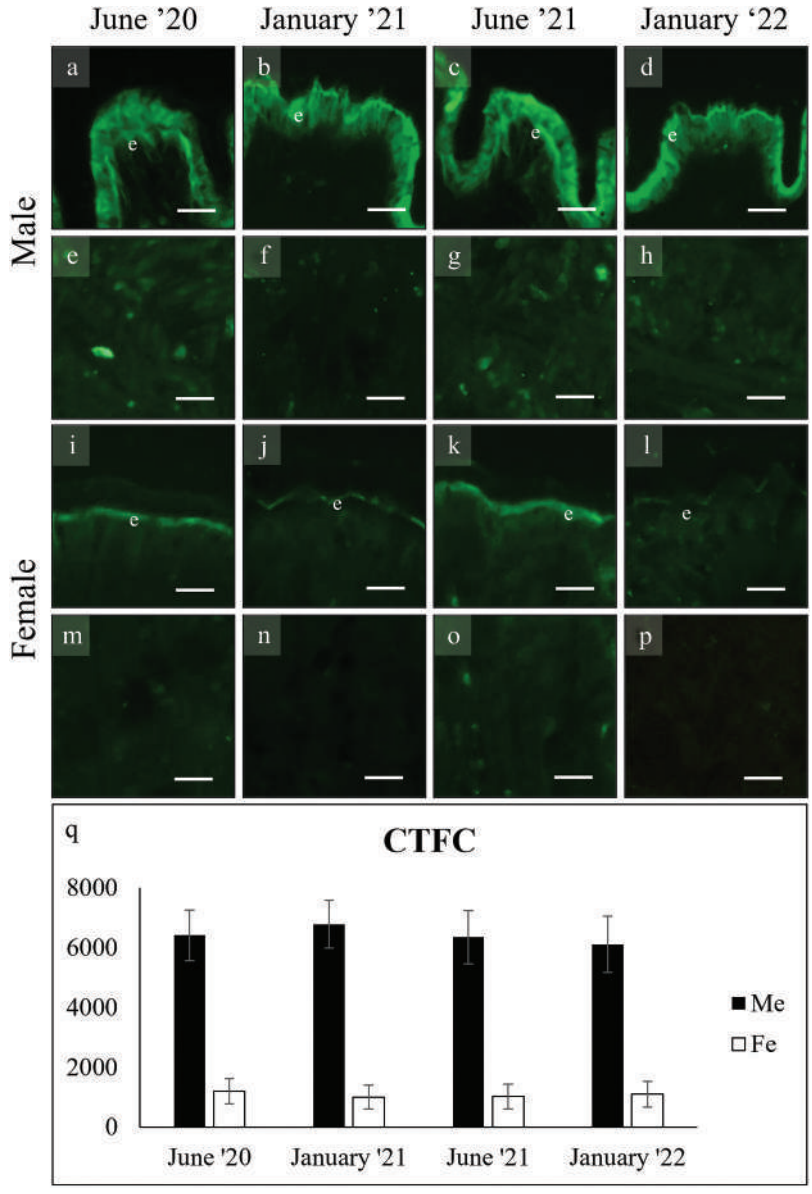

Figure 8. WGA binding of secretory cells in the foot of male (a-h), and female (i-p) *C. gallina*. The top images (a-d for males; i-l for females) show the variation of secretion between summers (June) and winters (January) conditions in the surface cells (e). Surface cells and glycocalyx stain intensely in males and weakly in females. The bottom images (e-h for males; m-p for females) shows that subepidermal glands never bind. (q) Plot of mean CTFC values for male surface cells (black) and female surface cells (white). Values in males are high in each month, while in females they are significantly lower. Abbreviations: F, surface cell in female; Me, surface cell in male. Scale bars: 10 μm.

fucosylated residuals, while mannosylated residuals lack. In both sexes, a reduction in acidic and galactosaminylated residuals is observed in January, while fucosylated residuals do not vary along the seasons. Sulfated residuals in males have higher values in June and lower in January, while the opposite is seen in females.

The surface secreting cells in the foot present a higher diversity of residuals. Similar to the gills, a general decrease in residuals is observed in January for both sexes, except for sulfates that increase in the males only. Glycosaminylated and fucosylated residuals were abundant in the males only, the latter increasing in January, whereas mannosylated residuals in both sexes increase in June.

Similar to the surface cells, in the foot subepidermal glands, a general decrease in acidic residuals is

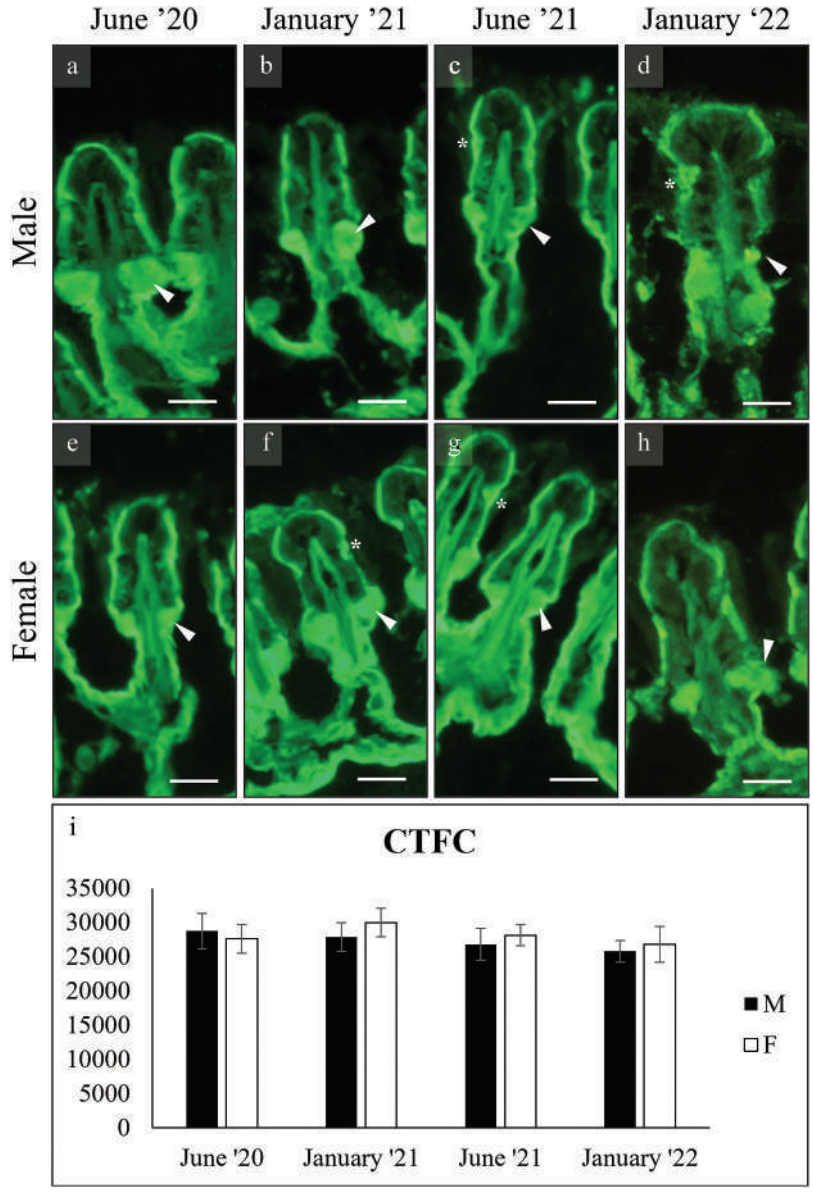

Figure 9. AAA binding of mucocytes (arrowhead) and glycocalyx (g) on the gill filaments of male (a-d) and female (e-h) *C. gallina*. AAA binds occasionally also to the frontal mucocytes (asterisk). Binding is intense for both sexes in each season. chitin skeleton of filaments (s) and to a second type of mucocyte (asterisk) in the frontal area. (i) Plot of mean CTFC values for gill main mucocytes in males (black) and females (white) shows high, almost constant values between sexes among seasons. Abbreviations: F, female; g, glycocalyx; M, male; *, frontal mucocyte. Scale bars: 10 μm.

observed in January, except for sulfates that increase in males. Mannosylated residuals are the only evidenced by lectin-binding and increase in both sexes in January.

In general, it would seem that in males, there is a higher variation than in females. The differences in mucins probably reflect functional requirements varying between sexes along the annual cycle. Since one of the main functions of mucus is surface protection and food entrapping and transport (Jørgensen 1996), we could hypothesize differences in energy allocation between sexes along seasons, in relation to environmental features and activities such as the reproduction. Temperature appears to be the main factor influencing the overall physiological responses and growth of striped Venus clams (Gaspar et al. 2004; Moschino & Marin 2006; Monari et al. 2007). *C. gallina* present an annual reproductive cycle characterized by one or more external emission of gametes (Romanelli et al. 2009) in which gametogenesis and spawning are strongly influenced by environmental parameters

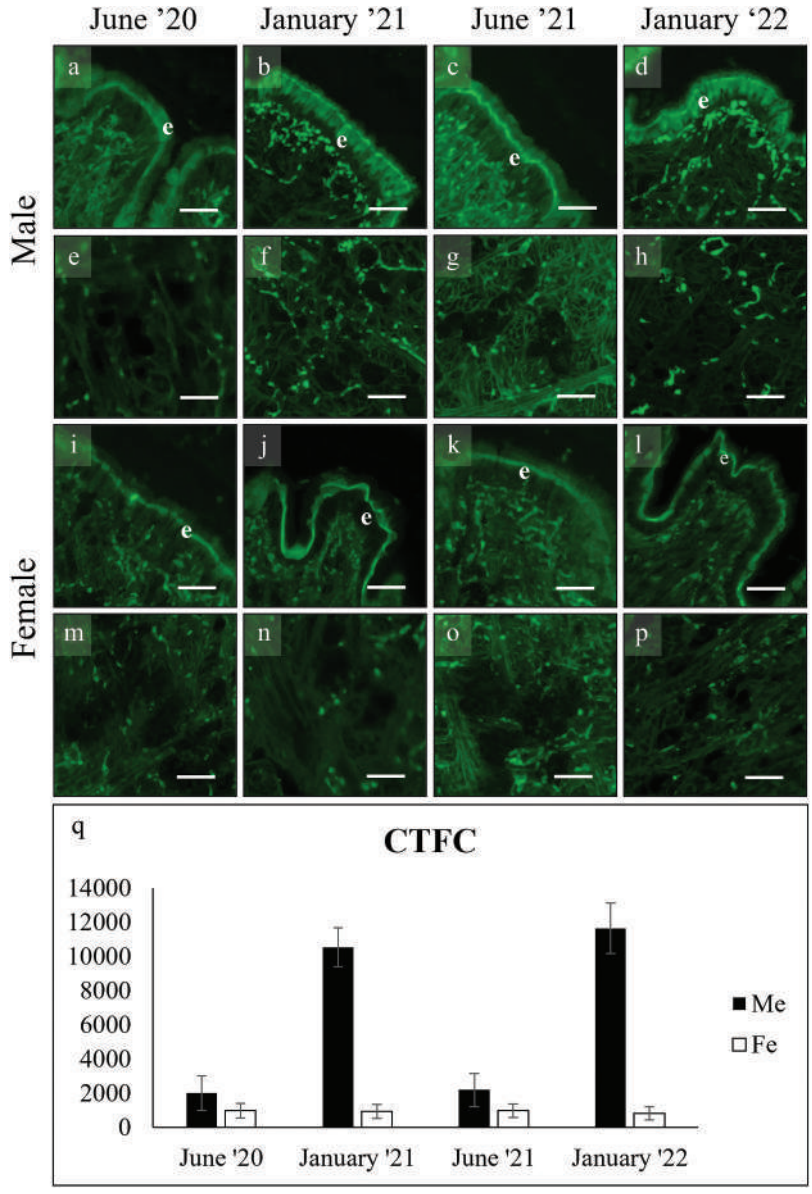

Figure 10. AAA binding of secretory cells in the foot of male (a-h), and female (i-p) *C. gallina*. The top images (a-d for males; i-l for females) show that surface cells (e) stain only in males in January. The glycocalyx stain intensely in males and weakly in females. The bottom images (e-h for males; m-p for females) shows that subepidermal glands never bind. (q) Plot of mean CTFC values for male surface cells (black) and female surface cells (white). Values in males are significantly high in January. Abbreviations: Fe, surface cell in female; Me, surface cell in male. Scale bars: 10 μm.

and food availability (Joaquim et al. 2014) and can vary greatly among individuals from the same area (Grazioli et al. 2022). Reproduction of clams spans from spring until late summer, with a peak in July (Nojima & Russo 1989). Increased respiration rates are observed during the reproductive period in another species of the Veneridae family, *Ruditapes decussatus*. (Urrutia et al. 1999).

Our histological observations on gonads indicated that in January, gametogenesis is more advanced in males than in females, thus it could be hypothesized that males have higher energetic requirements and synthesis of more complex mucins in this period, although secreted in lesser amounts. The lower variation observed in females could reflect more constant energetic requirements throughout the annual cycle since ovogenesis appears to span over longer periods with respect to spermatogenesis, although spawning is synchronized.

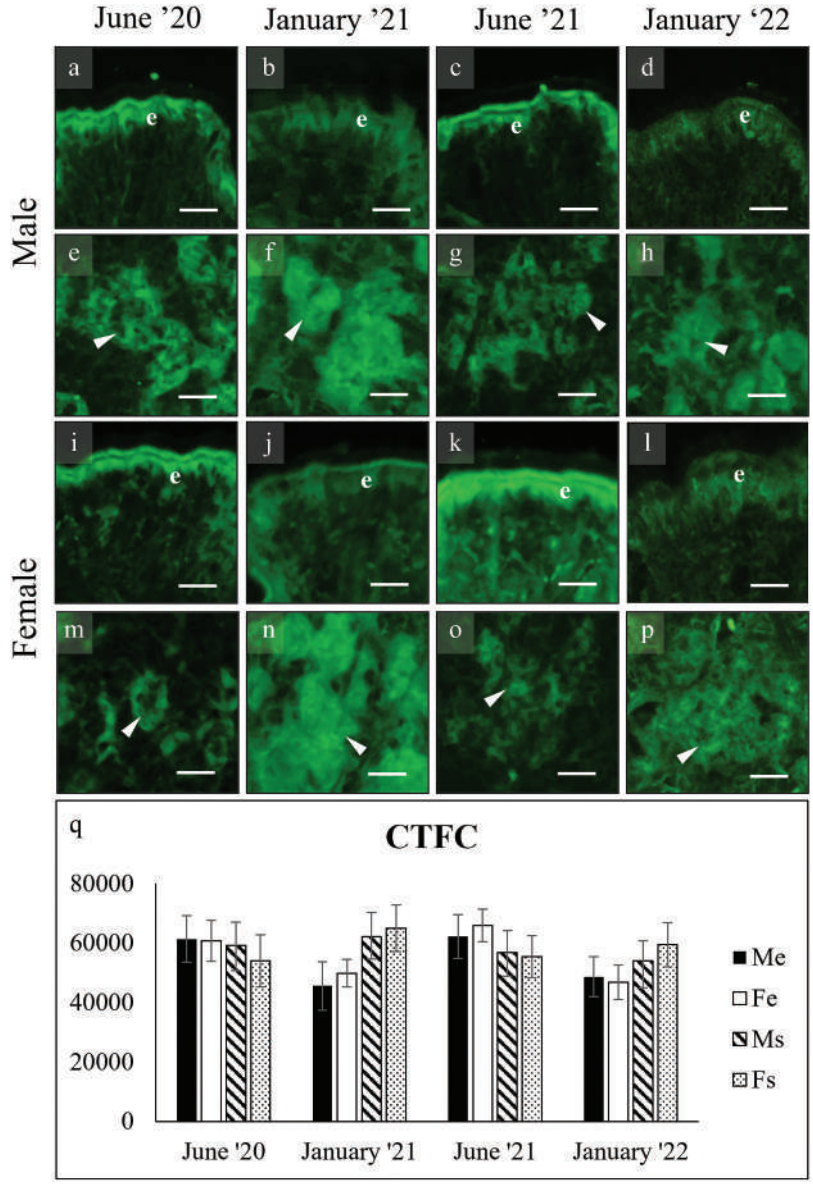

Figure 11. ConA binding of secretory cells in the foot of male (a-h), and female (i-p) *C. gallina*. For each sex, the top images (a-d for males; i-l for females) show the variation of secretion between summers (June) and winters (January) conditions in the surface cells (e), whereas the bottom images (e-h for males; m-p for females) shows the stain of subepidermal glands (arrowheads) in the same periods. (q) Plot of mean OD values for male surface cells (black), female surface cells (white), male subepidermal cells (striped) and female subepidermal cells (dotted). In the surface cells summer values (June 20 and June 21) are significantly higher than the corresponding winter ones (January 21 and January 22), whereas the opposite is seen in the subepidermal cells. Abbreviations: Fe, surface cell in female; Fs, subepidermal cell in female; Me, surface cell in male; Ms, subepidermal cell in male. Scale bars: 10 μm.

In conclusion, the present paper demonstrates extensive qualitative and quantitative variation of mucins secreted by the Venus clam depending on cell types, sex and season. It is necessary to consider these sources of variability in considering *C. gallina* a model for ecophysiological and toxicological studies, as well as in evaluating the status of populations exploited in fisheries. Further studies are necessary to elucidate the complex interactions between environmental features, biological activities and mucin secretion.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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