The adhesion of homogenized fat globules to proteins is increased by milk heat treatment and acidic pH: Quantitative insights provided by AFM force spectroscopy

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Abstract

The rheological properties and microstructure of dairy gels involve the connectivity between milk fat globules (MFG) and casein micelles that is affected by technological processes such as milk homogenization and heat treatment. The underlying mechanisms require further quantification of the interactions at the nanoscale level to be fully understood and controlled. In this study, we examined the adhesion of homogenized MFG to milk proteins and evaluated the role of ultra-high temperature (UHT) heat treatment and pH. The combination of physico-chemical analysis, rheology and microscopy observations at different scale levels associated to atomic force microscopy (AFM) force spectroscopy were used. AFM experiments performed at the particle scale level showed that adhesion of individual homogenized MFG to milk proteins (1) is increased upon acidification at pH 4.5: 1.4 fold for unheated samples and 3.5 fold for UHT samples, and (2) is enhanced by about 1.7 fold at pH 4.5 after UHT heat treatment of milk, from 176 pN to 296 pN, thanks to highly-reactive heat-denatured whey proteins located at the surface of MFG and caseins. The increased inter-particle adhesion forces accounted for more connected structures and stiffer UHT milk acid gels, compared to unheated-milk gels. Using a multiscale approach, this study showed that heat treatment of milk markedly affected the interactions occurring at the particle's surface level with consequences on the bulk structural and rheological properties of acid gels. Such findings will be useful for manufacturers to modulate the texture of fermented dairy products through the tailoring of heat-induced complexation of proteins and the connectivity of homogenized MFG with the protein network. This work will also contribute in a better understanding of the impact of process-induced changes on the digestibility and metabolic fate of proteins and lipids.

Keywords: Milk fat globule surface, Whey protein aggregate, Homogenization, Interface, Emulsion, Protein network, Acid dairy gel, Processed-induced changes, Atomic force microscopy

1. Introduction

The transformation of milk into dairy products involves many mechanical (e.g. homogenization), thermal (e.g. pasteurization, ultra-high temperature) and chemical (e.g. acidification) treatments. Such processes alter the structure of milk components, including milk fat globules (MFG) and casein micelles, as well as their surface properties and their connectivity through changes in the lipid–protein and protein–protein interactions. In consequence, the microstructure and rheological properties of dairy products are affected. However, the mechanisms underlying such structural and rheological changes remain poorly known at the particle scale level and require further investigations.

Milk is a natural oil-in-water emulsion composed of MFG (mean diameter 4 µm) dispersed in a suspension of proteins including the colloidal casein micelle assemblies (100 – 200 nm diameter; 80% w/w of milk proteins) and soluble whey proteins (WP; 20% w/w of milk proteins). The MFG consist of a core of triacylglycerol (TAG; 98% of the milk lipids) surrounded by a biological membrane, organized as a trilayer of polar lipids, cholesterol and glycoproteins, called the milk fat globule membrane (MFGM) (Lopez, 2011). The colloidal casein micelle assemblies are composed of κ-, β-, α_{s1}- and α_{s2}-caseins maintained by intermolecular hydrophobic interactions and calcium phosphate bridges. The glycosylated end of the peripheral κ-caseins forms an electronegative polymer brush that provides electrostatic and steric stability of the colloidal casein micelle assemblies at the milk pH, i.e. pH 6.7 (Dalgleish, 2011). Casein micelles are stable upon heating until 100°C (Dalgleish, 2011; Gaucheron, 2005), but readily precipitate into gels upon acidification, through neutralization of their surface charge at about pH 4.6 and collapse of their κ-casein brush (Gaucheron, 2005; Tuinier & de Kruif, 2002). WP are globular proteins whose native form is soluble across a wide range of pH. They consist of β-Lactoglobulin (β-Lg; 51% w/w of WP), α-Lactalbumin (α-La; 22% w/w of WP) and other minor proteins (Farrell et al., 2004). In contrast to casein micelles, WP are heat-sensitive. Heat-treatment above 70°C induces denaturation of the WP, leading to exposure of reactive-thiol groups and to WP aggregation through intermolecular hydrophobic interactions and formation of intermolecular disulfide bridges. In milk, the thiol-containing κ-caseins, and to a minor extent α_{s2}-caseins, can co-aggregate with the denatured WP, mainly β-Lg, inducing the attachment of a significant fraction of the WP aggregates onto the surface of casein micelles (Anema & Li, 2003b; Donato & Guyomarc'h, 2009; Vasbinder & de Kruif, 2003). The denatured WP do not stay soluble upon acidification and bear significant hydrophobicity. Authors showed that the presence of heat-induced WP aggregates

increases the firmness of acid skimmed milk gels (Morand, Dekkari, Guyomarc'h, & Famelart, 2012; Vasbinder & de Kruif, 2003). Therefore, heat treatment of milk can be used to modulate protein – protein interactions, and thus the texture of dairy products. However, scientific knowledge about the impact of heat-induced WP on the connectivity between homogenized MFG and the protein network in acid gels needs to be improved.

Heat treatment of milk (pasteurization, sterilization by ultra-high-temperature (UHT)) is performed in the dairy industry to ensure its microbiological safety and increase its shelf-life by decreasing the amount of bacteria, but also to modulate the texture of dairy products by inducing the denaturation of WP. Homogenization of milk is a mechanical process used to improve the physical stability of the emulsion by decreasing the size of MFG. The high shear stress applied during homogenization breaks up the 4-µm diameter MFG into MFG with diameters below 1 µm (Lopez, Cauty & Guyomarc'h, 2015). Furthermore, homogenization leads to the disruption of the MFGM and induces the adsorption of milk proteins, i.e. casein micelles and some native WP (below 5-7% of total adsorbed proteins), onto the newly formed surface of MFG (Lee & Sherbon, 2002; Ye, Anema, & Singh, 2008). In the dairy industry, homogenization of milk is combined with heat treatments. As an example, commercially available milk is commonly homogenized and heat-treated using pasteurization or UHT processing. In milks previously heated at temperatures above 70°C, the homogenization leads to adsorption of denatured WP onto the MFG surface that can reach 10-25% of the adsorbed proteins (García-Risco, Ramos, & López-Fandiño, 2002; Lee & Sherbon, 2002; Ye, Anema & Singh, 2008). Therefore, the introduction of a heating step prior to homogenization modifies the surface composition of homogenized MFG. Acidification of milk is a widely used chemical treatment that is involved in the production of dairy products, e.g. sour creams and yoghurts. Acidification is also a natural chemical mechanism occurring upon food digestion in the stomach that leads to the formation of a coagulum upon milk gastric digestion. The decrease in pH leads to the physical instability of the casein micelles alone or in interaction with the heat-sensitive WP, and to the formation of a protein network in which MFG can be more or less strongly entrapped depending on their surface properties. Upon gelation, homogenized MFG interact with the protein matrix, thereby increasing the gel's elastic modulus (Lopez & Dufour, 2001; Michalski, Cariou, Michel, & Garnier, 2002; van Vliet, 1988). Process-induced changes in the microstructure and rheological properties of milk at acid pH, such as those occurring after homogenization and heat-treatment, can have consequences on the mechanisms involved in MFG and protein digestion (Garcia, Antona,

Robert, Lopez & Armand, 2014; Liang, Qi, Wang, Jin, & McClements, 2017; Mulet-Cabero, Mackie, Wilde, Fenelon, & Brodkorb, 2019; Ye, Cui, Dalgleish, & Singh, 2017; Zhao, Du & Mao, 2019).

The impact of the main technological treatments involved in the dairy industry, i.e. heat-treatment, homogenization and acidification, on milk proteins and MFG has been widely investigated at the micro and macroscale (Michalski, Cariou, Michel, & Garnier, 2002; Lee and Sherbon, 2002; Jukkola and Rojas, 2017). However, information is lacking at the nanoscale on the role of the heat-denatured WP in modifying individual interaction between homogenized MFG and casein micelles in heated milk and acid gels. The main reason is the lack of experimental techniques allowing measurements directly from complex colloidal food systems at the relevant nanoscale level. Atomic force microscopy (AFM) is a well-adapted technique to image, with nanometer resolution, the surface topography of particles and to probe interaction forces with high sensitivity in the pN to nN range (Müller, Krieg, Alsteens, & Dufrêne, 2009). In recent years, AFM has gained growing interest in studying complex food systems (Gunning & Morris, 2018; West & Rousseau, 2018). In a previous study, we successfully used AFM force spectroscopy to investigate the impact of homogenization and pH on the adhesion forces between individual MFG and AFM probes functionalized with casein micelles (Obeid, Guyomarc'h, Francius, Guillemin, Wu, Pezennec, Famelart, Cauty, Gaucheron & Lopez, 2019). AFM experiments showed that casein-coated homogenized MFG interacted with higher adhesion forces to surrounding casein micelles, compared to native MFG covered by the MFGM with dense repulsive glycocalyx. The adhesion forces were increased at pH 4.5, with 109 pN for native MFG vs. 210 pN for homogenized MFG. This first study on MFG showed that AFM force spectroscopy is a promising technique to quantify adhesion forces at the particle scale level in complex systems such as food emulsions.

The present study was designed to determine whether the denaturation of WP and their interactions with casein micelles, induced by UHT heat treatment, could modulate the connectivity between homogenized MFG and the protein network formed at acidic pH. We examined the composition and surface properties of MFG and casein micelles, and quantified by AFM force spectroscopy the adhesion forces between individual homogenized-MFG and bulk proteins (i.e. casein micelles and WP). By using a multiscale approach, we related the adhesion forces measured at the individual particle level with the bulk characteristics of the milk acid gels, i.e. their microstructure and rheological properties.

144 # 2. Materials and methods

145 ## 2.1. Materials

146 Bulk tank raw whole bovine milk was provided by Gaec des Fougères (La Chapelle des
147 Fougeretz, France). Glucono-delta-lactone (GDL), Fast-Green FCF and Nile-Red fluorescent
148 dyes were purchased from Sigma-Aldrich (St Louis, USA). D(+)-sucrose was purchased from
149 VWR Chemicals (St Louis, USA). PIPES buffer was used as previously reported in Obeid et
150 al. (2019). PIPES buffer (1,4-piperazinediethane sulfonic acid) contained 10 mM PIPES, 50
151 mM NaCl, and 2 mM $CaCl_2$, all purchased from Sigma-Aldrich. The PIPES buffer was
152 adjusted to pH 6.7 or 4.5, using 1M NaOH or HCl respectively.

153

154 ## 2.2. Preparation of milk samples and isolation of casein micelles

155 Homogenized (H-) and UHT-homogenized (UHT-H-) milks, as well as native- and UHT-
156 skimmed milks were prepared from the same raw milk, using the technological facilities of
157 the INRA STLO dairy platform (Rennes, France). The pH of native milk, originally of 6.6 at
158 30°C, was adjusted to 6.5 in order to promote the interaction between denatured-WP and
159 casein micelles upon heat treatment (Anema & Li, 2003b; Vasbinder & de Kruif, 2003). To
160 prepare H-milk, the milk was heated to 50°C for 10 min and homogenized at 20 MPa (2 MPa
161 in the 2nd stage) with a high-pressure homogenizer Panda Plus 2000 (GEA Niro Soavi, Parma,
162 Italy; Flow: 9 L/h). To prepare UHT-H-milk, the milk was preheated from 40°C to 90°C (3
163 °C/sec) and from 90°C to 140°C (3 °C/sec) and immediately UHT heat treated at 140°C for 4
164 sec using an indirect UHT processing system (Microthermics UHT/HTST Lab 25 EDH,
165 Microthermics Inc., Raleigh, NC, USA). The UHT-milk was then rapidly cooled at 9 °C/sec
166 prior to homogenization at 20 MPa (2 MPa in the 2nd stage) with the high-pressure
167 homogenizer Panda Plus 2000. Sodium-azide (0.02% w/v) was added to the milk samples in
168 order to prevent bacterial growth. Milk samples were stored at 20 ± 1 °C and used within the
169 next three days after milk processing.

170 In order to isolate native- and UHT-casein micelles for AFM force spectroscopy experiments
171 (i.e. grafting of the AFM probes), the skimmed counterparts of the native and UHT-milks
172 were prepared. Raw milk at pH 6.5 was skimmed at 50°C using an Elecrem separator
173 (Vanves, France), then subjected to the same UHT heat treatment as described above for
174 whole milk. Native- and UHT-casein micelles were isolated from native- and UHT-skimmed
175 milks respectively to eliminate residual WP and other solutes. Briefly, skimmed milks were

diafiltered 5-fold using PIPES buffer pH 6.7 using a lab-scale Centramate tangential filtration system with 0.1 µm-cut-off membranes (Centramate cassettes, Pall Corporation, Saint Germain-en-Laye, France). Isolated casein micelles in PIPES buffer pH 6.7, with a final concentration of about 20 $g.kg^{-1}$, were aliquoted and stored at -20°C until use for AFM force spectroscopy experiments.

2.3. Denaturation rate of the whey proteins

The total nitrogen (TN), non-casein nitrogen (NCN) and non-protein nitrogen (NPN) contents were determined by the Kjeldahl method (IDF Standard 20B, 1993; ISO 8968-1, 2014; ISO 8968-4, 2016; ISO 17997-1, 2004), for native milks, H-milks, UHT-H-milks and skimmed milks, prior to or after UHT heat treatment. Nitrogen content was converted into equivalent protein content using 6.38, 6.25 and 6.19 as conversion factors for TN, NCN and NPN, respectively (Karman & Van Boekel, 1986). The amount of native WP was calculated as [Native WP] = [NCN] - [NPN]. Proteins that precipitate at pH 4.6, i.e. essentially caseins but also the denatured WP in heated samples, were calculated as [CN] = [TN] - [NCN]. From this, the respective amounts of denatured WP and caseins in [CN] of the heated samples, as well as the rate of WP denaturation, were calculated knowing [Native WP] and [CN] of the unheated milk reference.

2.4. Washing of homogenized milk fat globules

For the determination of the interfacial protein composition by gel electrophoresis and for AFM experiments, MFG were isolated from the milk aqueous phase to remove un-adsorbed proteins according to a method adapted from Patton & Huston (1986). Briefly, H- or UHT-H milks were warmed to 50°C, then 10 g of milk was mixed with 5 g of PIPES buffer pH 6.7 containing 50% w/w of sucrose. Then, in 50 mL plastic centrifuge tubes, 15 g of the treated milk were delivered under 30 g of a solution of PIPES buffer pH 6.7 containing 5% w/w of sucrose. The tubes were centrifuged at 2500 *g* for 30 min in order to form a layer of washed homogenized MFG at the top of the tubes. The protocol used for native MFG has been previously described (Obeid, Guyomarc'h, Francius, Guillemin, Wu, Pezennec, Famelart, Cauty, Gaucheron & Lopez, 2019).

2.5. Gel electrophoresis

After washing of MFG, the centrifuge tubes were frozen then cleaved just below the washed MFG layer. Collected MFG were diluted twice in SDS 1% w/w and centrifuged at 2500 *g* for 30 min to recover the desorbed interfacial proteins in the lower phase. Interfacial proteins desorbed from native-, H- and UHT-H-MFG as well as the proteins in isolated native- and UHT-casein micelle samples were quantified by micro-Kjeldahl method and analyzed by SDS-PAGE. The different protein samples were diluted in denaturing and reducing buffer containing 0.5 M Tris-HCl pH 6.8, 20% glycerol, 2% SDS, containing 0.5% bromopherol blue and 40 mM dithiothreitol (a reducing agent that breaks down the disulfide linkages responsible of the aggregation of heat-denatured WP) for 1 h at 37°C. As an alternative, in non-reducing conditions, protein samples were diluted in a similar buffer, but without dithiothreitol, to keep the disulfide bonds. Each diluted protein sample was then loaded on a sample well (10 µg of protein/well) on top of a precast 4-20% polyacrylamide gel electrophoresis (mini-protein TGX precast gel, Bio-Rad Laboratories, Dublin, Ireland). Electrophoresis was carried out at a constant current of 200 V for 45 min. Gels were rinsed with distilled water and were stained with Bio-safe Coomassie G250 stain (Bio-Rad Laboratories, Dublin, Ireland). A molecular weight (MW) marker (kit Precision Plus Protein Standards 10-250 kD, Bio-Rad Laboratories, Dublin, Ireland) was employed for MW calibration. The gels were scanned by an Image Scan II gel imager using the LabScan software (GE Healthcare, Velizy Villacoublay, France).

2.6. Size and zeta-potential measurements

The size distribution of MFG and the hydrodynamic diameter (D_h) of casein micelles were determined by laser light scattering (Mastersizer 2000, Malvern Instruments, Orsay, France) and by dynamic light scattering (NanoSizer-ZS, Malvern Instruments, Orsay, France), respectively, as detailed in Obeid et al. (2019). Briefly, for MFG size distribution measurements, samples were diluted in ultra-pure water in the absence or presence of 1% SDS to check for possible flocculation of processed MFG. To determine the D_h of casein micelles, samples were diluted 100 fold v/v in PIPES buffer at pH 6.7. Measurements were carried out at 20°C.

The electrophoretic mobility of MFG and casein micelles was measured at 20°C using a Zetasizer Nano ZS (Malvern Instruments, Orsay, France), after dilution of the samples to 1/1000 v/v in PIPES buffer at pH 6.7 or 4.5. The apparent ζ -potential was deduced using the Henry's equation and the Smoluchowski approximation.

All measurements, size and ζ –potential, were performed in triplicate and expressed as mean with standard deviation. Analyses of variance (ANOVA) were performed using the General Linear Model procedure of Statgraphics Plus version 5 (Statistical Graphics Corp., Englewood Cliffs, NJ). Differences between the sample means were compared at the 5% level of significance using Fisher's least significance difference test.

2.7. Rheological measurements

The visco-elastic properties of H-milks and UHT-H-milks were measured as a function of pH as previously described in Obeid et al. (2019). Rheological measurements were carried out at 50°C by small amplitude oscillatory rheology using a DHR-2 instrument (TA Instruments, Guyancourt, France) with coaxial cylinders. The storage modulus, G' (in Pa), was monitored as function of the pH, upon addition of 1.65% w/w GDL in milk samples, at a frequency of 1 Hz and 1% strain. The gelation pH was established as the intercept of a linear extrapolation of the rapidly rising storage modulus G' with the pH axis. The final G' value was taken at pH 4.5. Measurements were performed in triplicate and expressed as mean with standard deviation. ANOVA was performed using the General Linear Model procedure of Statgraphics Plus version 5. Differences between the sample means were compared at the 5% level of significance using Fisher's least significance difference test.

2.8. Microscopy experiments

2.8.1. Transmission electron microscopy

Transmission electron microscopy (TEM) was used to examine the homogenized milk and the UHT-homogenized milk. Briefly, H-milk and UHT-H-milk samples were solidified in agar 1.5% (w/v), cut into 1 mm pieces, fixed overnight with 25 $g.kg^{-1}$ glutaraldehyde in 0.1 M Na-cacodylate and post-fixed for 1h in 1% osmium tetroxide. The pieces were then dehydrated through increasing ethanol concentrations, infiltrated and embedded in Epon resin mixture (Electron Microscopy Sciences), then finally polymerized at 60°C for 24 h. Ultra-thin sections were cut using a Leica ultra-microtome and stained with 4% uranyl-acetate for 1h. Sections were observed with JEOL-1400 TEM microscope operating at 120 kV. TEM images were recorded on camera Gatan Orius SC-1000.

2.8.2. Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) experiments were performed with an inverted Eclipse-TE2000-C1si confocal microscope (NIKON, Champigny sur Marne, France) to investigate the microstructure of milks and acid gels, as described in Obeid et al. (2019). A He–Ne laser operating at 543 nm wavelength excitation, a laser diode operating at 642 nm and a x100 (NA 1.4) oil immersion objective were used. The TAG core of MFG was stained with Nile-Red fluorescent dye (exc. 543 nm). The proteins were stained using Fast-Green FCF (exc. 642 nm). After labeling, the samples were kept at 20°C for 30 min prior to CLSM observations. For observations at acid pH, the fluorescently-labeled milk samples were acidified by adding GDL (1.65 % w/w), incubated at 50°C and then observed when the milk gel reached pH 4.5.

An inverted CLSM Zeiss LSM 880 microscope (Carl Zeiss SAS, Marly Le Roi, France) was used to observe the modified AFM probes, using a laser at 561 nm (spectral detection range: 560 - 601 nm). AFM probes were incubated overnight at 4°C with fluorescent α_{s1}-caseins conjugated to Rhodamine-B-IsoThioCyanate (RITC) at 10 mg/mL in PIPES buffer pH 6.7 in PIPES buffer. The observations were performed using a x63 oil immersion objective.

2.8.3. Atomic force microscopy imaging and force spectroscopy

AFM experiments, imaging and force spectroscopy, were carried out at 22 ± 1°C using an MFP-3D Bio AFM (Asylum Research, Oxford Instruments, Santa Barbara, CA, USA).

Washed H-MFG or UHT-H-MFG, suspended to 1:10 in PIPES buffer pH 6.7, were immobilized on Isopore polycarbonate track-etched Millipore filters of 0.6 µm pore size by gently passing the solution through the filter membrane using a syringe, until blockage (**Fig. 1-A**). The filter membrane was gently rinsed with buffer, in order to remove loosely immobilized MFG. The filter membrane was then attached inside a Petri dish using a double-sided adhesive tape, and immersed in PIPES buffer pH 6.7.

Imaging of immobilized MFG was performed in PIPES buffer, using silicon MSNL probes (Bruker Nano Surfaces, Santa Barbara, CA - nominal spring constant of 0.01 $N.m^{-1}$) through the force-mapping mode, with a scan rate of 1Hz on 10 x 10 μm^2 surface and at least 64 × 64 pixels. The adhesion force measurements were performed through the force-mapping mode by using casein micelles-modified AFM probes prepared as follows. AFM probes with attached 5-µm borosilicate glass particles, coated with gold and modified with NH_3 groups (Novascan, Ames, Iowa, USA; nominal spring constant of 0.01 $N.m^{-1}$) were used. The AFM

probes were incubated overnight at 4°C in the dispersions of isolated native or UHT-casein micelles (20 $g.kg^{-1}$; thawed aliquots were left to equilibrated at 20°C for at least 1h before experiments prior to use to ensure complete rehydration and ionic balance of the casein micelles). The AFM probes were then washed and kept in PIPES buffer pH 6.7 until use. The successful grafting of caseins on the AFM probes was validated by CLSM experiments using fluorescent caseins (**Fig. 1-B**).

Fig. 1-A illustrates the two experimental set-ups that have been adopted to measure the adhesion forces between individual homogenized MFG and casein micelles, without or with a UHT heat treatment of the milks. On one hand, AFM measurements were performed between H-MFG and native casein micelles grafted on AFM probes (**Fig. 1-A-left**). On the other hand, measurements were performed between UHT-H-MFG and UHT-casein micelles grafted on AFM probes (**Fig. 1-A-right**). For each sample, adhesion force measurements were first performed in PIPES buffer at pH 6.7. Then, the PIPES buffer at pH 6.7 was replaced by PIPES buffer at pH 4.5 and the set-up was incubated for 30 min at this pH before starting again the force measurements. The force maps were recorded on 50 x 50 μm^2 surface and 30 x 30 pixels with an applied force of 500 pN, a probe velocity of 1 $\mu m.s^{-1}$ and a contact time of 1 sec. The cantilever spring constant was determined prior to each AFM force spectroscopy experiment using the thermal noise calibration method (Lévy & Maaloum, 2001). At least 3 independent experiments were performed to measure the adhesion forces in each experimental set-up at pH 6.7 then pH 4.5.

Interactions between casein micelles-modified AFM probes and MFG produce specific adhesion peaks in the retraction force curves as reported in Obeid et al. (2019) and illustrated in **Fig. 1-C**. In each experimental condition, force curves showing non-specific adhesive events or deformed baseline were discarded (Le et al., 2013). Force curves with adhesion forces below 45 pN were considered as noise. Force curves evidencing specific adhesion were analyzed in order to determine the maximal adhesion force (F_{adh}) and rupture distance (D_{adh}) as illustrated in **Fig. 1-C**. The AFM experimental data were statistically analysed using the Wilcoxon rank-sum test adapted for non-normally distributed data. Indeed, the distributions of F_{adh} or D_{adh} were not Gaussian, because the measured adhesion involves objects (casein micelles, homogenized MFG) whose dimensions are not themselves normally distributed. Differences were considered statistically significant when P value < 0.001.

3. Results and discussion

3.1. UHT heat treatment leads to the denaturation of whey proteins in milk

Compositions of the protein fractions (i.e. caseins, native- and denatured-WP) in the different milk samples are presented in **Fig. 2-A**. Casein fraction represented about 80% w/w of the total protein content in the different samples, in agreement with the literature (Dalgleish, 2011; Farrell et al., 2004). The percentage of native-WP was about 20% w/w in native milks and skimmed milks, in agreement with the literature (Ahmad et al., 2008; Farrell et al., 2004). Denatured-WP were not present in milk samples that were not subjected to UHT heat treatment (i.e. native milk, H-milk and skimmed milk samples). In contrast, about 16% w/w of total milk proteins corresponded to denatured-WP in milk samples that were subjected to UHT heat treatment, i.e. UHT-skimmed milk and UHT-H-milk (**Fig. 2-A**). This corresponds to about 80% w/w of the total WP in each sample. These results showed that, as expected, UHT heat treatment of milk induced significant denaturation of the WP (Anema & McKenna, 1996; Oldfield, Singh, Taylor, & Pearce, 1998).

3.2. Surface properties of MFG and casein micelles in homogenized and UHT-homogenized milks

3.2.1. UHT heat treatment alters the surface protein composition of both MFG and casein micelles

Fig. 2-B (left part) shows the SDS-PAGE patterns corresponding to the protein compositions of native- or UHT-casein micelles that have been isolated from native- or UHT-skimmed milk respectively. The protein patterns of native casein micelles corresponded exclusively to caseins (i.e. α-, β-, κ-caseins) between 25-37 KDa. In contrast, UHT-casein micelle protein patterns showed, in addition to caseins, the presence of β-Lg (~18 KDa). Under non-reducing conditions, the band corresponding to β-Lg and that of κ-caseins were not present in the gel electrophoresis, indicating that the UHT heat treatment applied to milk induced aggregation of β-Lg with the κ-caseins. This is in agreement with the literature (Anema & Li, 2003a; Donato & Guyomarc'h, 2009; Vasbinder & de Kruif, 2003).

Fig. 2-B (right part) shows the SDS-PAGE patterns corresponding to the proteins present at the surface of MFG in native milk, H-milk and UHT-H-milk. The surface proteins of native-MFG corresponded exclusively to MFGM proteins, i.e. the xanthine oxidase (154 KDa), butyrophiline (67 KDa), PAS 6 (50 KDa), PAS 7 (47 KDa) and the mammary derived growth inhibitor (14 KDa) already described in previous reports (Fong, Norris, & MacGibbon, 2007; Mather, 2000; Ye, Singh, Taylor, & Anema, 2002; Obeid et al., 2019). In contrast, the surface

proteins of H-MFG and UHT-H-MFG, corresponded mainly to caseins. Also, bands corresponding to β-Lg and α-La have been identified mainly on the surface UHT-H-MFG and to a lesser extent (i.e. faint bands) on the surface of unheated H-MFG. Under non-reducing conditions, the surface protein patterns of UHT-H-MFG were lacking the bands corresponding to WP, mainly β-Lg, and to κ-casein (**Fig. 2-B**). This result evidenced that UHT heat treatment induced the formation of complexes between casein micelles and WP proteins, and that these protein aggregates were adsorbed at the surface of UHT-H-MFG. These results confirmed i) the adsorption of milk proteins (caseins and WP, mainly β-Lg and α-La) to the surface of MFG during the homogenization process, and ii) that heat-denaturation of WP induced by UHT heat treatment favors their co-adsorption with casein micelles onto the MFG surface (Lee & Sherbon, 2002; Ye, Anema & Singh, 2008).

The protein analysis of the particles, MFG and casein micelles, showed that i) homogenization of milk leads to a partitioning of the casein micelles and the non-denatured WP between the surface of MFG and the aqueous phase of milk; ii) UHT heat treatment applied to milk leads to the formation of protein complexes between casein micelles and WP. These complexes are located both in the aqueous phase of milk and at the surface of homogenized MFG.

3.2.2. UHT heat treatment alters the size, the morphology and the ζ-potential of casein micelles

The hydrodynamic diameter D_h of the native- and UHT-casein micelles was of 173 ± 2 and 256 ± 5 nm, respectively. Therefore, the UHT heat-treatment induced significant increase ($P < 0.001$) of the D_h of casein micelles, by about 80 nm. The morphology and size of the native casein micelles observed using TEM (**Fig. 2-C**) were in agreement with the literature (Marchin, Putaux, Pignon, & Léonil, 2007). In contrast, TEM images of UHT-casein micelles (**Fig. 2-C**) revealed protruding filaments at the surface of casein micelles that may correspond to the binding of protein aggregates composed of denatured-WP and κ-casein. The colloidal casein micelle assemblies were therefore affected by UHT heat treatment. This is in agreement with the early study of Mottar et al. (1989) who showed using electron microscopy that UHT heat treatment produced long and numerous filaments of denatured-WP at the casein micelle surface.

The ζ-potential of casein micelles was affected by UHT heat treatment and by the pH. At pH 6.7, UHT-casein micelles exhibited a significantly lower ($P < 0.05$) negative charge of -13.5 ±

0.7 mV compared to a value of -15.6 ± 0.4 mV measured for native-casein micelles (**Table 1**). This was due to the presence of denatured-WP at the UHT-micelle surface (**Fig. 2-C**) (Anema & Klostermeyer, 1996; Donato & Guyomarc'h, 2009). Decreasing the pH from 6.7 down to 4.5 affected the ζ-potential values of the casein micelles: -2.3 ± 0.5 mV for non-heated casein micelles and -2.7 ± 0.5 mV for UHT-casein micelles ($P > 0.05$). This decrease in the negative charge was due to the protonation of the acido-basic groups, e.g. phosphate and carboxylic residues of casein micelles (Gaucheron, 2005). The low negative charge of the casein micelles at acidic pH reduces the electrostatic repulsions between casein micelles and is responsible for the formation of a protein network in acid gels.

3.2.3. Homogenization of milk alone or combined with UHT heat treatment alter the size and ζ-potential of fat globules

After homogenization at 20 MPa, H-milk and UHT-H-milk exhibited similar size distributions of MFG with two major peaks centered at about 0.7 and 0.2 µm (results not shown). As expected, the small size of MFG in both homogenized milks (below 1 µm) compared to that of native MFG (4 µm) was due to the high shear stress applied during the homogenization process (Obeid et al., 2019; Lopez, Cauty, & Guyomarc'h, 2015; Michalski & Januel, 2006). Homogenization alone or combined with UHT heat treatment of milk did not induce MFG aggregation. TEM observations of H-milk and UHT-H-milk showed a layer of proteins adsorbed at the surface of MFG, mainly casein micelles identified due to their spherical morphology. This is in agreement with the chemical analysis of surface proteins (**Fig. 2-B**) and previous TEM observations of H-milk (Obeid et al., 2019).

UHT heat treatment of milk significantly ($P < 0.05$) affected the ζ-potential values of MFG. The ζ-potential values of MFG were -17.3 ± 0.5 mV in H-milks and -15.4 ± 0.2 mV in UHT-H-milks (**Table 1**). Similarly, the ζ-potential of washed MFG prepared for AFM force spectroscopy experiments was significantly ($P < 0.05$) more negative for washed H-MFG compared to washed UHT-H-MFG (-15.6 ± 0.9 mV vs. -14.3 ± 0.5 mV; **Table 1**). This is due to the presence of denatured-WP co-adsorbed with casein micelles at the surface of UHT-H-MFG. These results are in agreement with the difference in ζ-potential values that have been measured between native and UHT-casein micelles (**Table 1**). Decreasing the pH from 6.7 down to 4.5 affected the ζ-potential values of the MFG (**Table 1**). At pH 4.5, the negative ζ-potential values of MFG, homogenized or UHT-homogenized, were almost neutralized as a result of protonation of the acido-basic groups of casein micelles adsorbed at the MFG

surface. At acidic pH, the low negative charge of the homogenized MFG reduces the electrostatic repulsions between MFG (MFG-MFG interactions) and between the MFG and the surrounding casein micelles. This favors hydrophobic attractive interactions that are the basis of the building of milk gels.

3.3. Adhesion forces between homogenized milk fat globules and casein micelles are enhanced by acidic pH and UHT heat treatment

AFM topography images of washed and immobilized H-MFG and UHT-H-MFG, recorded in buffer at pH 6.7, are shown in **Fig. 3-A**. The H-MFG formed a continuous layer of juxtaposed particles in both samples. Similar AFM topography images were obtained upon acidification to pH 4.5 (results not shown).

Fig. 3-B, D show examples of adhesion force maps measured between the casein micelles-modified AFM probes and immobilized H-MFG or UHT-H-MFG, at pH 6.7 and pH 4.5. The measured adhesion forces, F_{adh} values, ranged from few pN to few nN. Force curves showing no adhesive events (below 45 pN) are represented as dark-blue pixels in the force maps. The percentage of force curves showing significant adhesion events was of 47 and 57 % for the adhesion between native casein micelles and H-MFG at pH 6.7 and 4.5, respectively. This percentage was significantly higher between UHT-casein micelles and UHT-H-MFG than between their unheated counterparts, with values of 71 and 94 % at pH 6.7 and 4.5, respectively. The adhesion forces increased upon acidification to pH 4.5 in both experimental set-ups as evidenced by the increase in the proportion of green and orange pixels in the force maps recorded at pH 4.5 (**Fig. 3-D**) compared to those measured at pH 6.7 (**Fig. 3-B**).

The retraction force curves showed adhesion events between the casein micelles grafted on the AFM probes and the H-MFG or the UHT-H-MFG, at pH 6.7 (**Fig. 3-C**) and at pH 4.5 (**Fig. 3-E**). The adhesion events were successfully fitted with the Worm-Like Chain (WLC) model that describes the mechanical stretching response of polypeptides under a pulling force, with supplementary insights on the flexibility of molecules during multiple interactions which is likely to occur when using colloidal probes (Friedrichs et al., 2013; Marszalek & Dufrêne, 2012; Francius et al., 2009; Obeid et al., 2019). The adhesion events were therefore the signature of the stretching of polypeptides molecules, i.e. the casein micelles alone or coated with heat-denatured WP, located both at the surface of MFG or grafted on the AFM probes. This is in agreement with our previous study on adhesion forces between homogenized casein-coated MFG and casein micelles (Obeid et al., 2019).

From the retraction force curves, the adhesion forces F_{adh} and the maximal rupture distance D_{adh} were analyzed as described in **Fig. 1-C**. The median F_{adh} between unheated casein micelles and unheated H-MFG increased nearly 1.4 fold, from 125 pN at pH 6.7 to 176 pN at pH 4.5 ($P < 0.001$; **Fig. 4-A**). Meanwhile, the median F_{adh} between UHT-casein micelles and UHT-H-MFG increased about 3.5 fold, from 85 pN at pH 6.7 to 296 pN at pH 4.5 ($P < 0.001$; **Fig. 4-A**). Concomitantly, the maximal rupture distance (D_{adh}) also increased significantly ($P < 0.001$) upon acidification of the experimental media in both unheated and UHT conditions (**Fig. 4-B**). Such an increase in F_{adh} and D_{adh} values upon acidification is in agreement with previous results obtained with native MFG and homogenized MFG (Obeid et al., 2019). This increase in Dadh when pH is decreased should be related to stronger attractive interaction and less electrostatic repulsive forced between the components present at the surface of MFG and the proteins grafted on the AFM probe. Furthermore, for each pH condition, the D_{adh} was shorter between UHT-casein micelles and UHT-H-MFG than between unheated casein micelles and unheated H-MFG. At pH 4.5 for instance, the median D_{adh} were respectively 110 and 151 nm (**Fig. 4-B**; $P < 0.001$). Furthermore, only at pH 6.7, the median F_{adh} between unheated casein micelles and unheated H-MFG was significantly higher (125 pN) than that between UHT-casein micelles and UHT-H-MFG (85 pN (**Fig. 4-A**), even though the percentage of adhesive events was lower in the first case (47% vs. 71% respectively).

3.4. Microstructure and acid gelation behavior of the milks as a function of pH

The CLSM observations performed at neutral pH showed that the MFG and milk proteins in both H-milk and UHT-H-milk samples were homogeneously dispersed in the aqueous phase (**Fig. 5**). This results from the electrostatic repulsion between the negatively charged homogenized MFG and the casein micelles (**Table 1**). CLSM observations performed after acidification of milk samples to pH 4.5 (**Fig. 5**) showed in both samples that the casein micelles aggregated and formed a protein network where the MFG were fully entrapped, as the result of the presence of casein micelles adsorbed on their surface (**Fig. 2 B**). This is in agreement with previous CLSM observations of acid homogenized milk gels (Lopez, Cauty & Guyomarc'h, 2015; Michalski et al., 2002; Obeid et al., 2019). Moreover, **Fig. 5** shows that the acid gels formed by H-milk displayed clustered network, while that formed by UHT-H-milk showed more branched network with higher connectivity. This is in agreement with the literature (Kalab, Emmons, & Sargant, 1975; Lucey, Munro, & Singh, 1998). As a conclusion,

the UHT heat treatment of milk affected the microstructure of the milk acid gels as compared to unheated H-milk.

Rheological measurements were performed as a function of the decrease in pH in both H-milk and UHT-H-milk (**Fig. 6**). The transition from liquid to gel state was obtained when the storage modulus G' became higher than the loss modulus G" (results not shown). The pH of H-milk decreased more slowly compared to that of UHT-H-milk. The time necessary to reach the gelation pH was of 24.3 ± 1.6 and of 6.4 ± 0.6 min for H-milk and UHT-H-milks, respectively. In addition, the gelation pH was significantly ($P < 0.001$) lower for H-milk (pH = 4.9 ± 0.1) compared to UHT-H-milk (pH = 5.2 ± 0.1). This is in agreement with previous studies showing that the presence of heat-denatured WP/κ-casein aggregates increases the gelation pH from about 4.9 to 5.1-5.3 and decreases the gelation time of skimmed milk acid gels (Krasaekoopt, Bhandari, & Deeth, 2003). In addition, the final G' value of milk acid gels at pH 4.5 was about 7 fold higher in UHT-H-milk gels compared to H-milk gels (136.2 ± 12.7 Pa vs. 18.7 ± 2.8 Pa; $P < 0.001$; **Fig. 6**). As a conclusion, the UHT heat-treatment of homogenized milk increased the stiffness of the acid gels as compared to non-heated homogenized milk, which is consistent with previous studies (Guyomarc'h, Queguiner, Law, Horne, & Dalgleish, 2003; Krasaekoopt et al., 2003; Lucey & Singh, 1997).

3.5. Impact of milk heat treatment and pH: multiscale link between surface properties of homogenized MFG and casein micelles, nanoscale adhesion forces, microstructure and rheological properties

In this study, we showed that the process-induced changes in the surface properties of MFG and casein micelles were markedly sensitive to the pH (**Table 1**) with consequences on the adhesion between particles (**Fig. 3 and 4**), on the microstructure (**Fig. 5**) and on the rheological properties of milk acid gels (**Fig. 6**), as summarized **Figure 7**. These results require further discussions.

At neutral pH, both the casein micelles and protein-coated homogenized MFG were negatively charged in H-milks and UHT-H-milks (**Table 1**). Thus, under physiological conditions, electrostatic repulsions prevented contact between homogenized MFG and the casein micelles located in the surrounding aqueous phase, as shown in confocal images (**Fig. 5**). However, in our AFM force spectroscopy experiments, casein micelles grafted on the AFM probe and immobilized homogenized MFG were forced into contact during the

recording of approach curves (**Fig 1-A**). In the absence of UHT heat treatment, a median adhesion force F_{adh} of 125 pN occurred between native casein micelles, either attached to the AFM probes or covering the surface of H-MFG (**Fig. 4-A**). Native casein micelles are flexible assemblies with low secondary and tertiary structures (Holt, Carver, Ecroyd, & Thorn, 2013). Once in contact, they interact through hydrogen bonds, hydrophobic and van der Waals interactions and can rearrange up to optimized interaction (Dalgleish, 2011). In absence of heat treatment, native WP do not contribute to this caseins – caseins interaction (Lucey, Munro, & Singh, 1999). When the milks were UHT heat-treated before homogenization, the adhesion forces involved UHT-casein micelles with denatured-WP aggregates attached to their surface both grafted on the AFM probe and adsorbed at the surface of MFG (**Fig. 1-A & 2-C**). The ζ-potential measurements showed a decrease in the negative value (**Table 1**) due to the adsorption of WP induced by UHT heat treatment. Denatured-WP were therefore responsible of the significant ($P < 0.001$) decrease in the median adhesion forces F_{adh} from 125 pN to 85 pN (**Fig. 4-A**). The denatured-WP aggregates protruding from the casein micelle surface (**Fig. 2-C**) have been reported to decrease the flexibility (i.e. deformability) of the UHT-casein micelles (Donato & Guyomarc'h, 2009). This may be responsible for the decrease in the F_{adh} and D_{adh} measured between UHT-casein micelles and UHT-H-MFG at pH 6.7 compared to the same parameters measured during adhesion between native-casein micelles and H-MFG. Therefore, at pH 6.5-6.7, the presence of denatured-WP aggregates at the surface of the casein micelles in UHT samples somewhat further prevented adhesion between individual casein micelles, already stabilized by electrostatic and steric repulsions.

At acidic pH of 4.5, the low negative charges of casein micelles, WP aggregates and homogenized MFG, with or without UHT heat treatment (**Table 1**), lead to a reduction in the electrostatic repulsion between these components. In addition, casein micelles undergo several changes upon acidification to pH 4.5 mainly demineralization and the collapse of their κ-casein hairy layer (Gaucheron, 2005). The formation of a protein network in which homogenized MFG were entrapped in the casein strands was observed in CLSM images, with stranded protein structures due to high hydrophobic interactions between denatured-WP in the UHT-H-milk gel (**Fig. 5**). AFM experiments showed that the adhesion forces F_{adh} between casein micelles and homogenized MFG significantly ($P < 0.001$) increased upon acidification in unheated samples (from 125 pN to 176 pN; 1.4-fold) and UHT heat-treated samples (from 85 pN to 296 pN; 3.5-fold) (**Fig. 4-A**). The denatured-WP aggregates present both at the surface of the UHT-casein micelles and adsorbed at the surface of UHT-H-MFG enhance the

surface hydrophobicity and increase the number of adhesive sites on the surface of UHT-casein micelles (Donato & Guyomarc'h, 2009; Jean, Renan, Famelart, & Guyomarc'h, 2006). The denatured-WP aggregates are responsible for significantly ($P < 0.001$) higher adhesion forces measured between UHT-casein micelles grafted on the AFM probes and on the UHT-H-MFG (median F_{adh} = 296 pN), compared to the adhesion between native casein micelles and H-MFG (median F_{adh} = 176 pN) (**Fig. 4-A**). The results obtained at the individual particle level were coherent with rheological properties measured at the macroscopic scale, where the G' value recorded at pH 4.5 for UHT heat-treated homogenized milk was significantly ($P < 0.001$) higher than that of the unheated homogenized milk (**Fig. 6**). These results obtained at the nanoscale highlight the role of inter-particle interactions in the building of the macroscale rheological properties of the acid milk gels (**Fig. 7**). In a pioneer work, Uricanu et al. (2004) evidenced that the G' value of individual casein micelles, measured by AFM indentation, did not significantly vary as a function of pH. From this, they hypothesized that inter-particle interactions, rather than intra-particle ones, were responsible for the macroscopic texture of the acid milk gels. Our results now bring positive evidence to support this assumption, and show that measurement of particle-particle adhesion force at the nanoscale is predictive of the bulk texture of acid milk gels.

From a methodological point of view, this study and the previous one (Obeid et al., 2019) showed that AFM force spectroscopy provides new quantitative information into the interaction between individual MFG and proteins at the nanoscale level which govern at the macroscale level the microstructure and rheological properties of dairy acid gels. This work clarifies in light of science the assumptions made in the last decade about the physicochemical interactions between lipids and proteins in a complex food system such as milk. Also, this work opens new perspectives for a better understanding of the effect of the interfacial compositions on the pair-pair interactions between individual food objects that is involved in food product quality.

4. Conclusion

Full understanding of how technological processes impact the interactions between particles, e.g. lipid-protein or protein-protein interactions, that contribute to the microstructure and rheology of food products require further knowledge. In this paper, we studied for the first time at the nanoscale level the effect of UHT heat treatment of milk on the interactions between individual homogenized MFG and casein micelles, using AFM force spectroscopy.

We showed that homogenized MFG interact with the surrounding proteins at acidic pH and that they are active fillers in a protein network. UHT heat treatment of milk increased the adhesion forces measured at pH 4.5 between protein-coated homogenized MFG and the proteins grafted on AFM probes. This was due to the presence of highly reactive heat-denatured WP attached to the casein micelles. Denatured-WP in UHT-homogenized milks ensures higher hydrophobic adhesion forces i) between casein micelles in the protein strands forming the acid gel, and ii) between the casein micelles adsorbed at the surface of H-MFG and those in the protein network. This leads to the formation of highly connected and stiffer UHT heat-treated acid gels compared to that formed by unheated homogenized-milks. This study provides new quantitative information at the nanoscale level through AFM force spectroscopy on the effect of technological processes (homogenization, heat treatment, acidification). Such findings will be useful for the dairy industry to modulate the texture of fermented dairy products, for example yoghurts, through the tailoring of heat-induced complexation of proteins and the connectivity of homogenized MFG with the protein network.

Acknowledgements

Sameh Obeid's postdoctoral fellowship was funded by CNIEL (French Dairy Interbranch Organization, Paris, France) in the Moon project (scientific coord. C. Lopez, INRA). The authors thank all the members of CNIEL's committee on dairy lipids for stimulating discussions about milk fat globules and dairy processing. Christelle Lopez warmly thanks Mr and Mrs Denoual from Gaec des Fougères for providing milk samples. The authors are grateful to the INRA STLO Platform (https://www6.rennes.inra.fr/plateforme_lait). Maryvonne Pasco and Jordane Ossemond (INRA-STLO, Rennes) are acknowledged for their contribution in gel-electrophoresis analysis of MFG surface proteins and CLSM observations of AFM tips, respectively. Grégory Francius (LCPME, CNRS, Nancy, France) and Jennifer Burgain (LIBio, Université de Lorraine, Nancy, France) are thanked for useful advice on analysis of AFM retraction force curves using the WLC model and on AFM colloidal probes, respectively. TEM images were taken at the Microscopy Rennes Imaging Center (University Rennes 1, France). The Asylum Research MFP3D-BIO atomic force microscope was funded by the European Union (FEDER), the French Ministry of Education and Research, INRA, Conseil Général 35 and Rennes Métropole.

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Table

Table 1. Zeta-potential values of milk fat globules (MFG) and casein micelles after milk homogenization (H) at 20 MPa, or after ultra-high temperature (UHT) heat treatment combined with homogenization at 20 MPa. The zeta-potential values have been determined at pH 6.7 and 4.5 in milk and in samples of washed MFG or casein micelles isolated from milk. ND: non-determined due to the precipitation of caseins.

Samples	ζ-potential (mV)	
	pH 6.7	**pH 4.5**
H-milk	-17.3 ± 0.5*	ND
UHT-H-milk	-15.4 ± 0.2*	ND
Washed H-MFG	-15.6 ± 0.9**	-4.7 ± 0.6
Washed UHT-H-MFG	-14.3 ± 0.5**	-4.0 ± 0.5
Native-casein micelles	-15.6 ± 0.4***	-2.3 ± 0.5
UHT-casein micelles	-13.5 ± 0.7***	-2.7 ± 0.5

Mean values calculated from 3 independent experiments. Analysis of variance: *, **, *** indicate significant differences with $P < 0.05$.

Figure caption

Fig. 1. (A) Schematic illustration, not to scale, of the AFM force spectroscopy experiments performed to measure the adhesion forces between casein micelles grafted to the AFM probes and homogenized milk fat globules (MFG) immobilized on filter membranes. The casein micelles and MFG were isolated from milks that have been either homogenized or heat treated at ultra-high temperature (UHT) and homogenized. AFM measurements were performed in PIPES buffer at 22°C. **(B)** Confocal laser scanning microscopy image showing fluorescently-labelled caseins successfully grafted on the AFM probe. **(C)** Example of AFM force curves where the maximal adhesion force F_{adh} and rupture distance D_{adh} are determined from the retraction curve.

Fig. 2. Chemical analysis and morphology of proteins. **(A)** Relative percentage of of milk proteins: caseins, native whey proteins (WP) or heat-denatured-WP in native milk, native skimmed milk, homogenized (H-) milk, and samples of milks after heat treatment at ultra-high temperature (UHT). **(B)** SDS-Page patterns of isolated native and UHT-casein micelles (CM) and of the interfacial proteins adsorbed on native-MFG, H-MFG and UHT-H-MFG. (*) indicates electrophoresis under non-reducing conditions. **(C)** Transmission electron microscopy images of native (left) and UHT heat-treated casein micelles (right).

Fig. 3. Atomic force microscopy (AFM) imaging and force spectroscopy experiments performed to measure the adhesion between milk proteins and milk fat globules (MFG) isolated from milks that have been either homogenized (H-MFG; left) or heat treated at ultra-high temperature and homogenized (UHT-H-MFG; right). **(A)** AFM topography images of MFG immobilized on filter membranes. **(B & D)** Adhesion force maps recorded at pH 6.7 and 4.5, using AFM probes modified with native- (left) or with UHT-casein micelles (right). The percentages are the rates of force curves with specific adhesion. **(C & E)** Examples of retraction force curves obtained from each force map recorded at pH 6.7 and 4.5. All experiments were performed at 22°C in PIPES buffer.

Fig. 4. Parameters extracted from retraction curves recorded by atomic force microscopy (AFM) force spectroscopy to examine the adhesion between casein micelles and milk fat globules (MFG) isolated from milks that have been either homogenized (H-MFG) or heat treated at ultra-high temperature and homogenized (UHT-H-MFG). Boxplots showing the distribution of **(A)** the maximal adhesion forces F_{adh}, and **(B)** the maximal rupture distance

D_{adh}, measured at pH 6.7 and 4.5. The number of analyzed individuals (n) is shown for each set of experiments. The bold lines in the boxes indicate the median values. (***) indicates significant differences, with $P < 0.001$, using the Wilcoxon test.

Fig. 5. Microstructure of milks and acid gels observed by confocal laser scanning microscopy (CLSM). CLSM images of milks at pH 6.5: (left) milk homogenized at 20 MPa; (right) milk heat-treated at ultra-high temperature (UHT: 140°C, 4 sec) and homogenized at 20 MPa. CLSM images of the acid gels obtained at pH 4.5. In the CLSM images, proteins are in green while the triacylglycerol core of fat globules is in red. Scale bars are 5 µm. Arrows indicate stranded structures in the acid gel formed from UHT heat-treated and homogenized milk.

Fig. 6. Evolution of the storage modulus G' as a function of the decrease in pH measured for homogenized milk (H-milk; blue) or milk that has been heat treated at ultra-high temperature and homogenized (UHT-H-milk; red). Analysis of variance: *** indicate significant differences with $P < 0.001$.

Fig. 7. Schematic representation, from nanoscale inter-particle forces to bulk properties, of the interactions between casein micelles and milk fat globules (MFG) in non-heated homogenized milk or ultra-high temperature (UHT) heat-treated and homogenized milk, with the consequences on the microstructure and rheological properties of the respective milk acid gels formed at pH 4.5. The green arrows represent the adhesion forces between homogenized MFG and casein micelles as determined by atomic force microscopy. CLSM images show the proteins (in green) and the MFG (in red) in the network formed at pH 4.5.

FIGURES

Figure 1

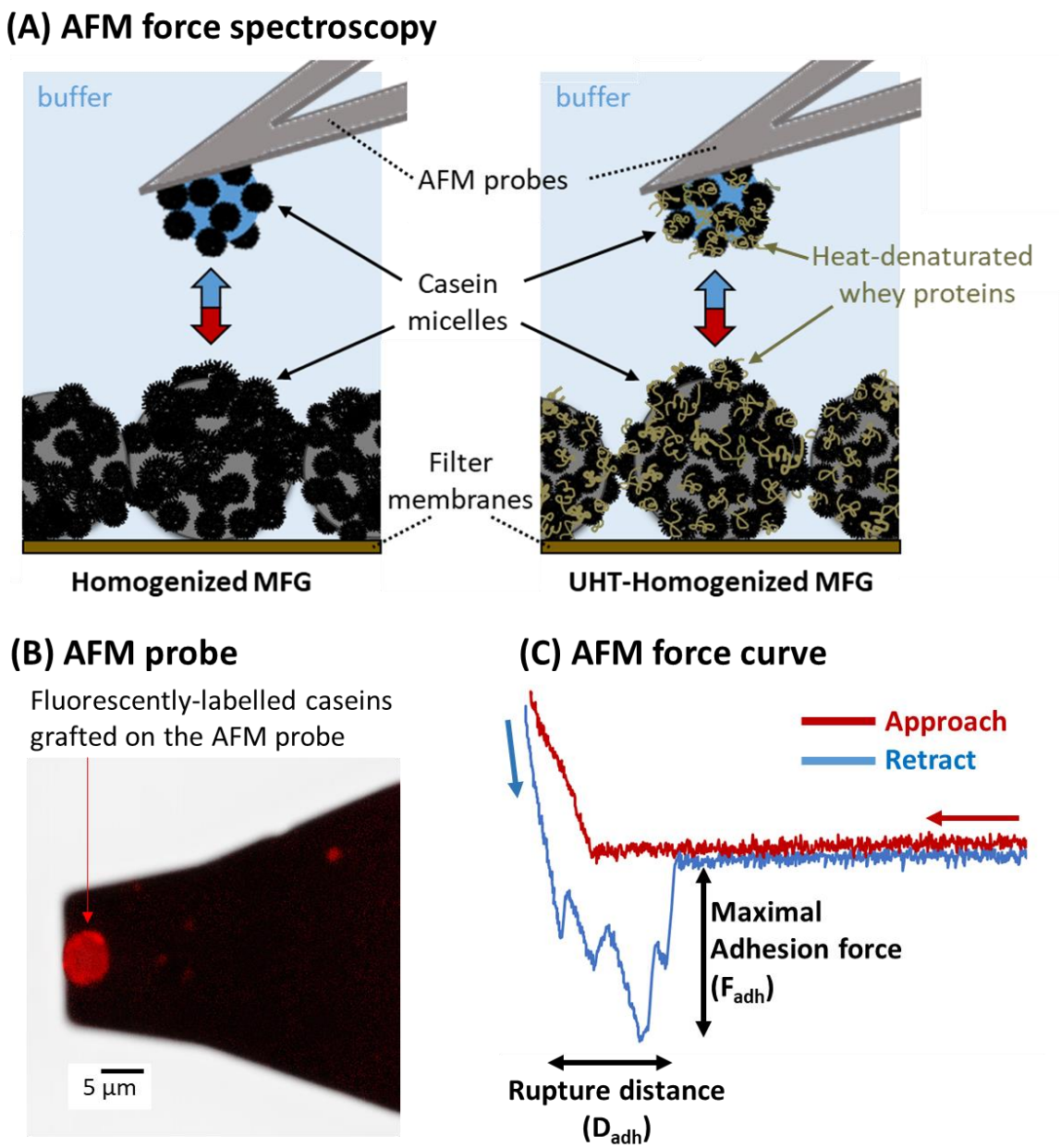


Figure 2

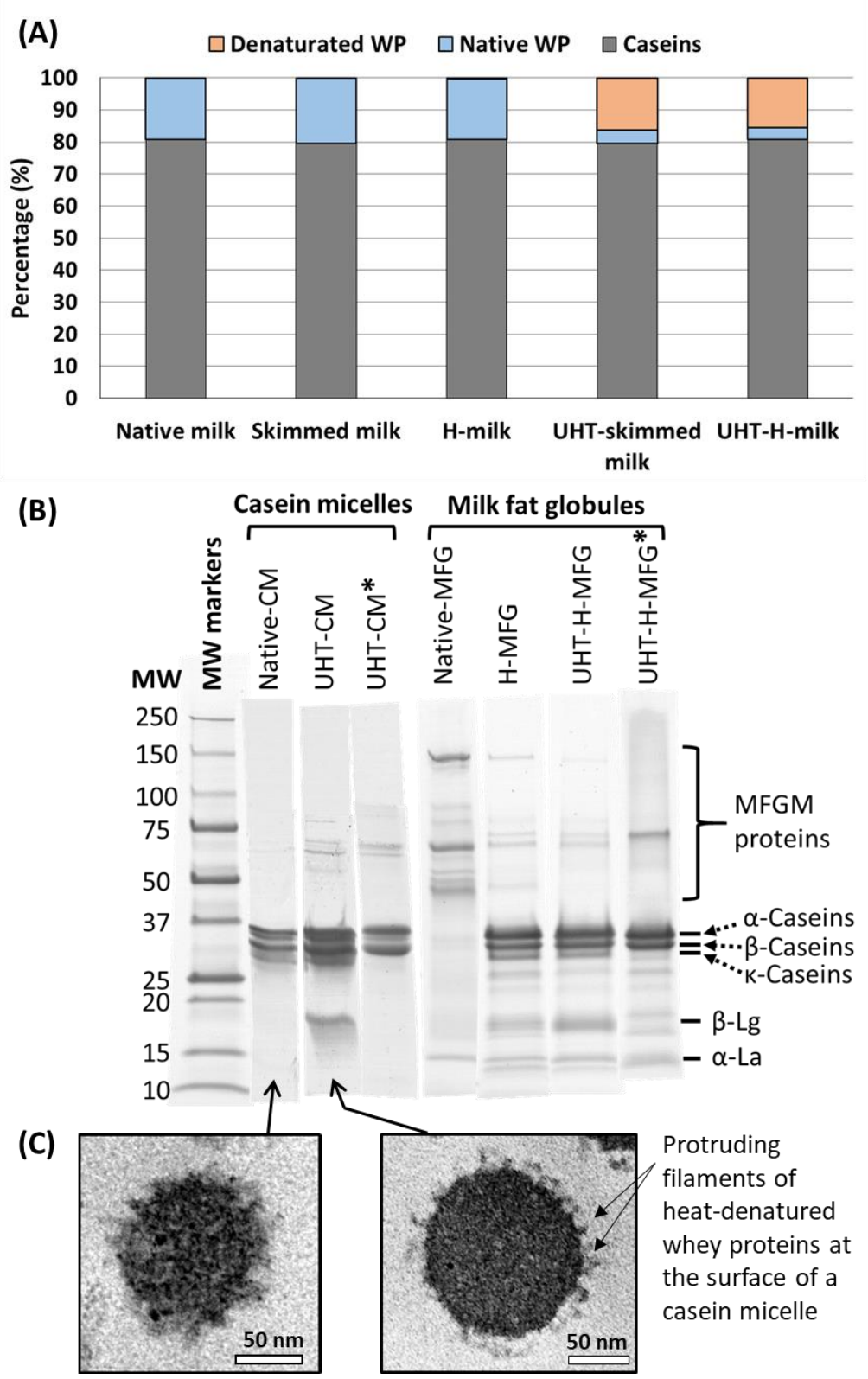
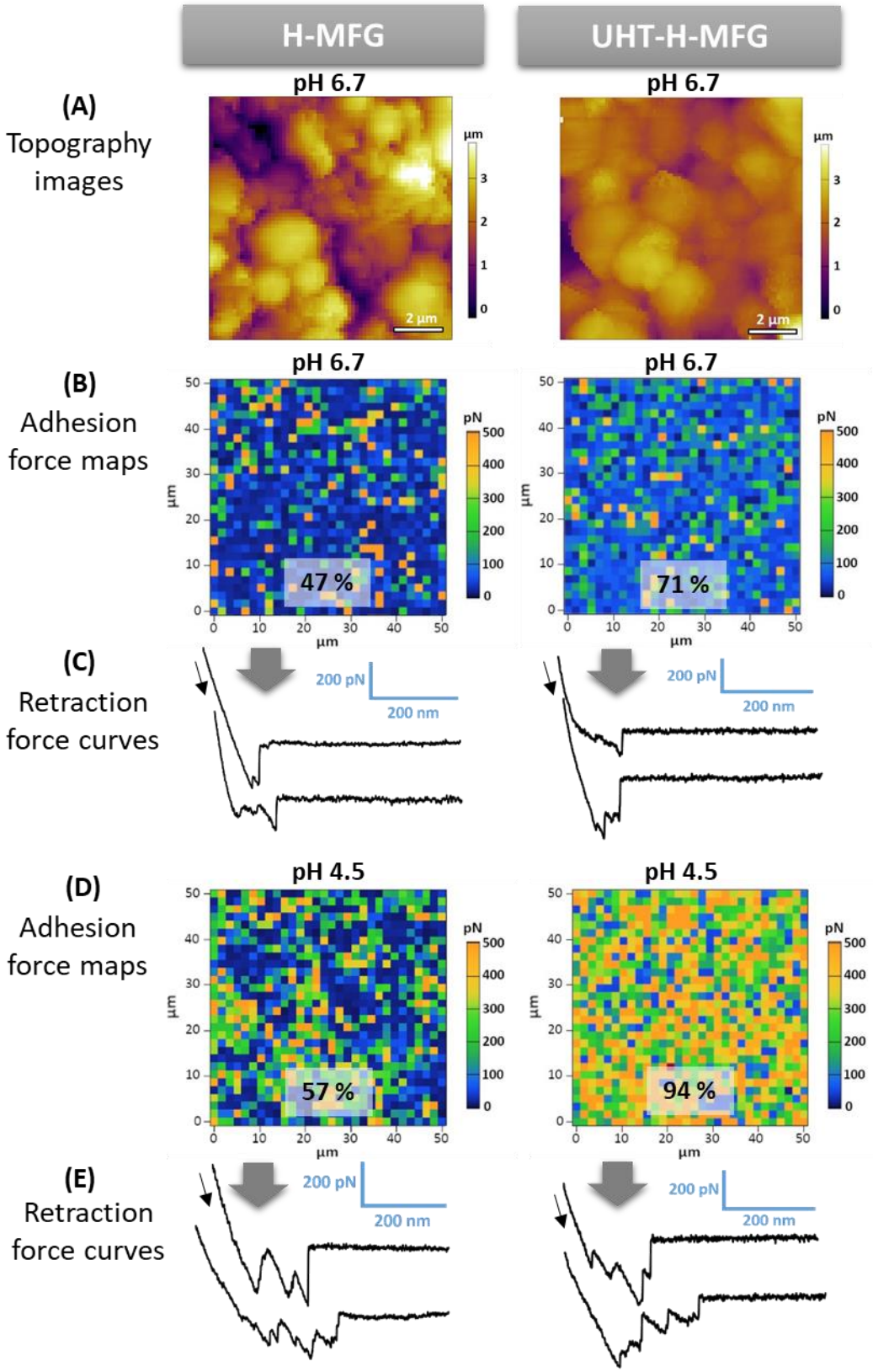
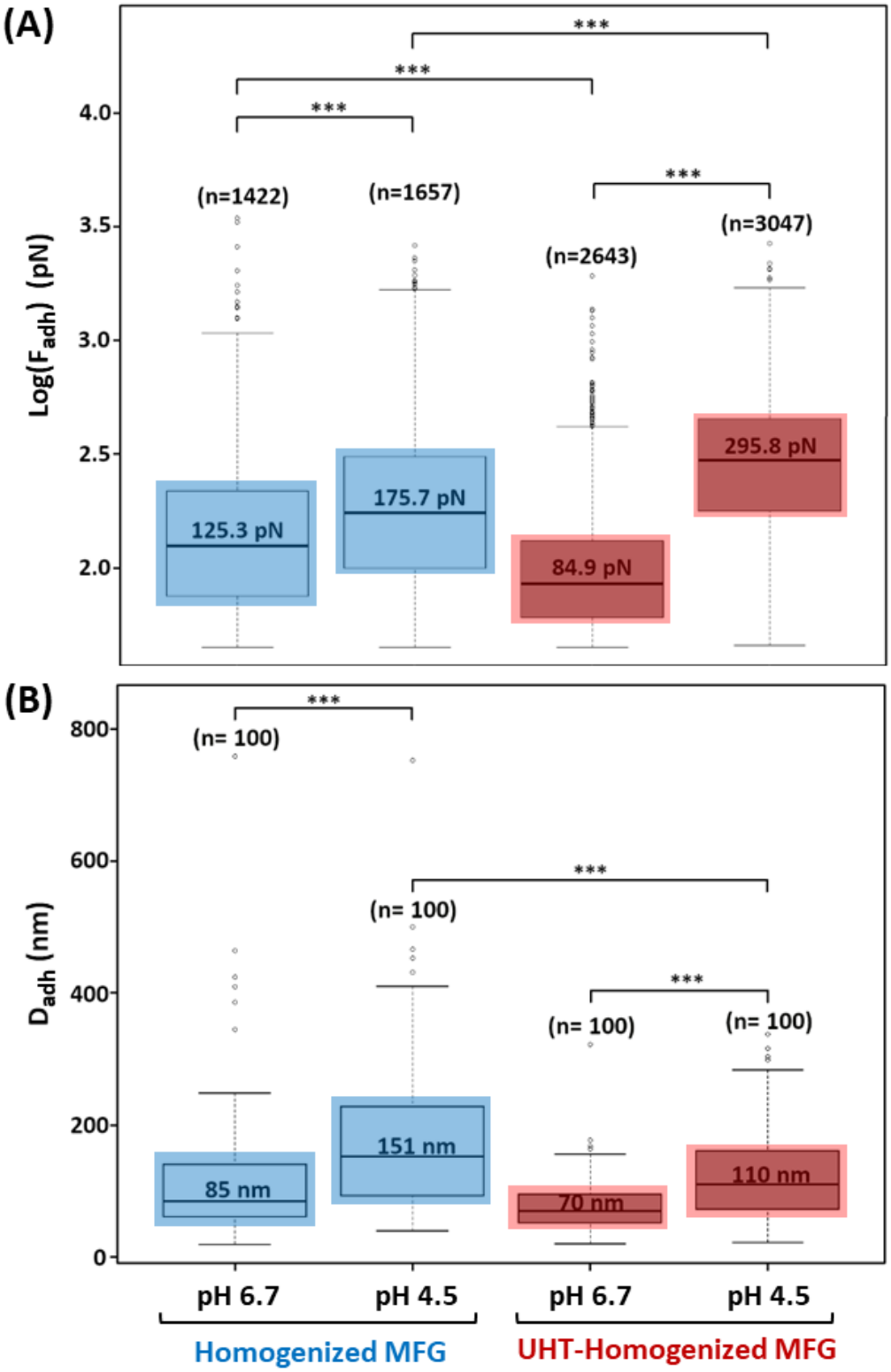

(A)
Denaturated WP
Native WP
Caseins
Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
Native milk
Skimmed milk
H-milk
UHT-skimmed milk
UHT-H-milk
(B)
Casein micelles
Milk fat globules
MW
MW markers
Native-CM
UHT-CM
UHT-CM*
Native-MFG
H-MFG
UHT-H-MFG
UHT-H-MFG*
250
150
100
75
50
37
25
20
15
10
MFGM proteins
α-Caseins
β-Caseins
κ-Caseins
β-Lg
α-La
(C)
50 nm
50 nm
Protruding filaments of heat-denatured whey proteins at the surface of a casein micelle

Figure 3


H-MFG
UHT-H-MFG
(A)
Topography
images
pH 6.7
pH 6.7
µm
2 µm
(B)
Adhesion
force maps
pH 6.7
pH 6.7
pN
47 %
71 %
(C)
Retraction
force curves
200 pN
200 nm
(D)
Adhesion
force maps
pH 4.5
pH 4.5
57 %
94 %
(E)
Retraction
force curves

901 **Figure 4**



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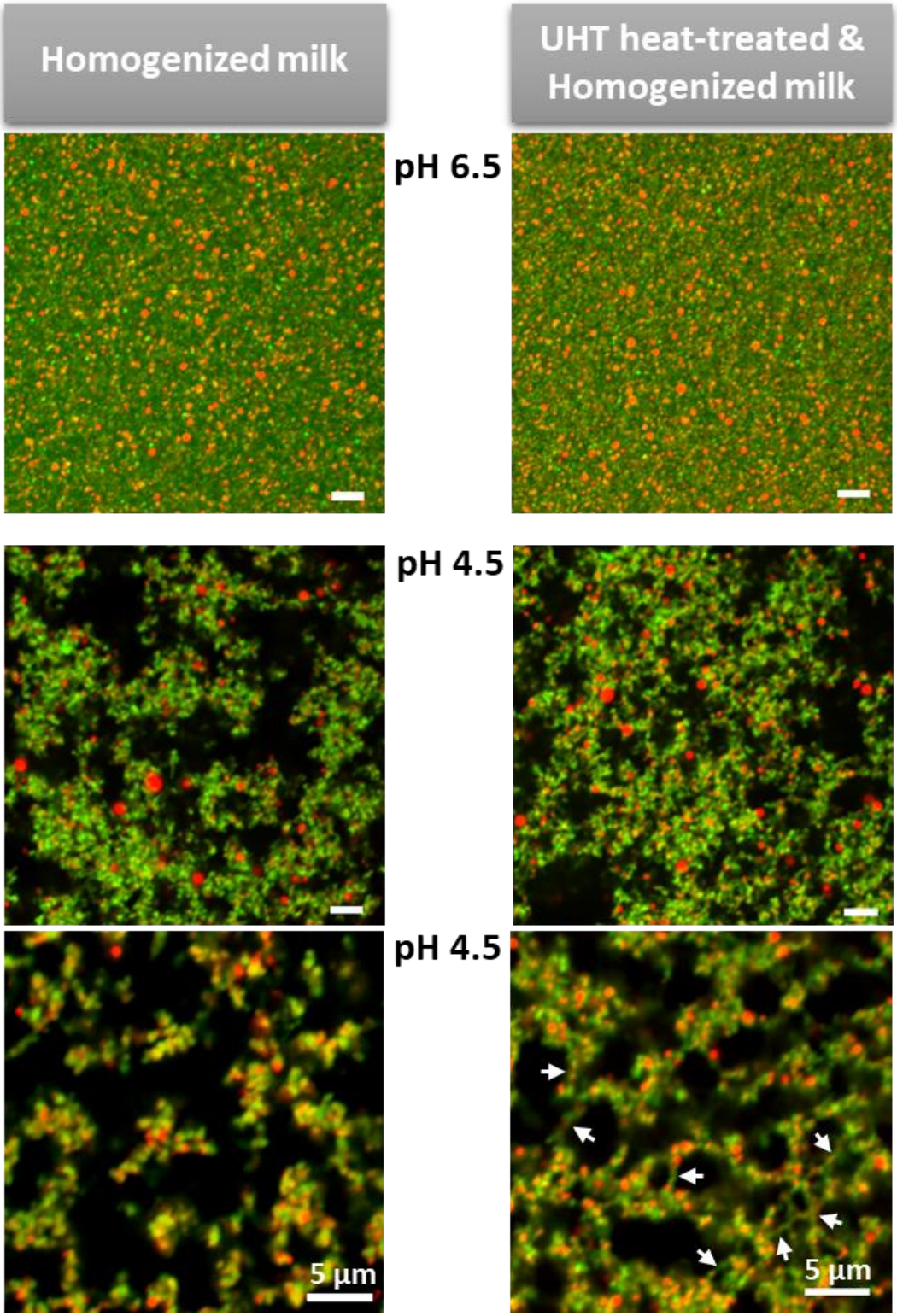
905 **Figure 5**



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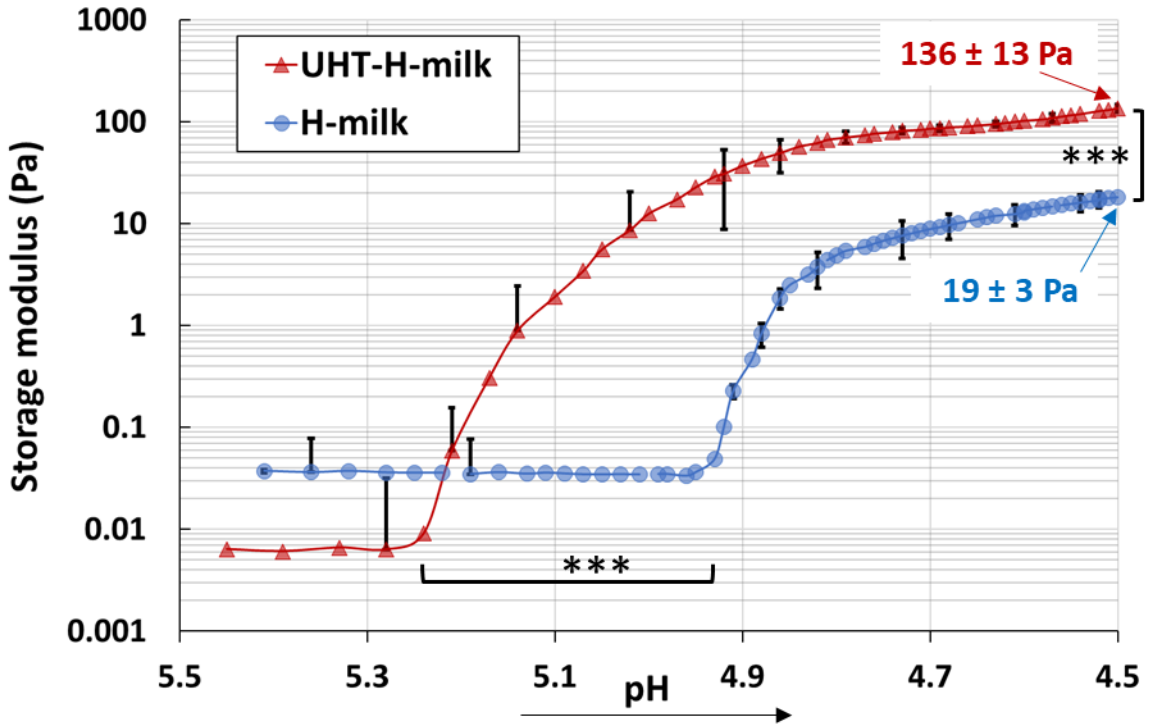
908 **Figure 6**



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915 **Figure 7**

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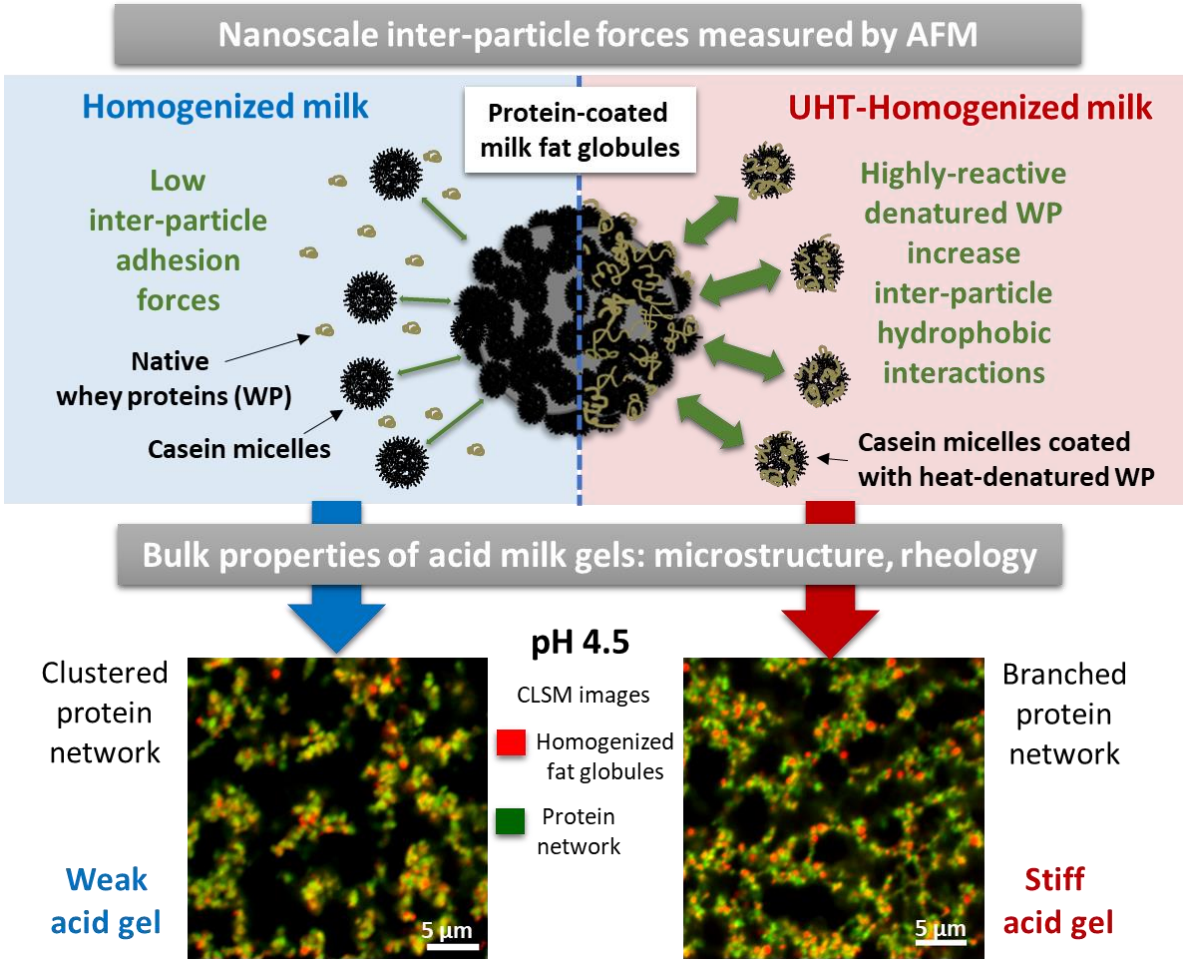

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WHAT'S IT LIKE OUT THERE? LANDSCAPE LEARNING DURING THE EARLY PEOPLING OF THE HIGHLANDS OF THE SOUTH-CENTRAL ATACAMA DESERT

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Abstract

It has often been assumed that a link exists between climate change and human dispersion during the initial peopling of the Atacama Desert. However, there is little understanding of how hunter-gatherers acquired and processed environmental information. We examine paleoenvironmental and archaeological data to propose that the early peopling of the highlands of the south-central Atacama was a gradual process modulated by landscape learning. Evidence suggests that initial occupations at the end of the Pleistocene were limited to intermediate altitude levels, where the ecological structure was more easily legible and productive. This allowed human groups to make use of general, transferrable landscape knowledge, based mainly on the hunting of wild camelids and the gathering of plant resources in azonal formations. However, the arid event of the Early Holocene led to successive episodes of abandonment and relocation to new areas, consolidating complementary land-use between the desert lowlands and the high puna. Two complementary strategies for the acquisition and transmission of information can be identified: (1) scouting as part of logistical hunting parties; and (2) information-pooling rooted in broad, flexible social networks. We conclude that in the face of uncertain conditions, hunter-gatherers invested more effort in learning the landscape and sharing environmental knowledge.

Keywords: Landscape learning, Highlands, Atacama Desert, Early peopling.

1. Introduction

The initial peopling of the highlands of the south-central Atacama (21-24ºS) is related to the "Tuina-Tambillo" phase (13,000 to 9500 cal BP). From early in this period, small hunter-gatherer bands developed a lifestyle well adapted to high-altitude deserts of the Salt Puna eco-region (Fig. 1). The development of a generalist technology, broad-spectrum subsistence and a seasonal mobility pattern enabled them to complement the wide variety of resources available in the different altitude levels of the Andes range (Núñez and Santoro, 1989; Núñez et al., 2002). Most authors agree that the Pleistocene-Holocene humidity peak played an important role in early human dispersal by offering more productive habitats. However, the spatial and temporal discontinuity of the archaeological record suggest a much more complex scenario. While some patches show occupation from an early date, others were abandoned, or only occupied later, while some areas were avoided completely. Clearly, it is not possible to establish a direct link between human dynamics and resource availability. Instead, a focus on "how" hunther-gatherers acquired and transmitted environmental knowledge could be an appropriate way to evaluate the rate of human dispersal and habitat preferences.

Many questions remain unanswered, but three are of particular interest for our case study: What prior knowledge existed? What had to be learned and what barriers did human groups face? What strategies were used to acquire landscape information? Landscape learning is a suitable approach for addressing these questions (Rockman, 2003, 2009), but not particularly easy to infer from the archaeological record (Kelly 2003). Mainly, because the acquisition and transmission of knowledge occur primarily in the social arena (Tolan-Smith, 2003). We will therefore examine the temporal and spatial variability of the archaeological record from a multi-

scale approach. The study of lithic technology provides an important line of evidence, as it allows us to explore the behavioural and cognitive dimension behind human dynamics in the landscape. We will show that populations dispersed progressively and selectively as this "Mars-like" landscape was learned. Complementary strategies to acquire and transmit information counteracted the partial uncertainty during human dispersal, as well as the negative climate fluctuations of the Early Holocene. In the long term, the new knowledge fostered deep changes in social organization, leading to a growing specialization in the high-altitude desert environment.

2. Landscape learning and environmental knowledge

Landscape learning is a staged process in which human groups acquire, transmit and store knowledge about the environment that they inhabit (Meltzer, 2003; Rockman, 2003, 2009). It encompasses a series of interrelated sub-processes occurring on different temporal and spatial scales, ranging from individual experience, through the intergenerational time of a local group, to adaptive radiations in human evolution. Although more emphasis has been placed on exploration and initial colonization, for hunter-gatherers learning the landscape is a critical and constant activity (Milne, 2014). In an immediate-return economy, even small disturbances in the climate and resource structure could have serious effects that must be anticipated and predicted. Besides, environmental cognition is a complex task mediated by mental models that are constantly evolving and being updated (Golledge, 2003), thus it is always imprecise and never completed (Meltzer, 2003). Foragers must therefore spend a large part of the time in acquiring and rectifying information during their daily social interactions (Fitzhugh et al., 2011). Landscape learning is thus a day-to-day task occurring in the social arena (Tolan-Smith, 2003), in which two aspects are vital: mobility and social networks. While the former takes individuals to places to observe, the second is the architecture through which information flows.

When a new territory is populated, the incoming population does not start from zero (Meltzer, 2003). If there are no other resident groups or even their traces to extract information, the knowledge acquired in their previous habitats can be good basis for generating expectations (Steele and Rockman, 2003), and often promotes exaptative advantages (Gamble, 1993). However, applying preconceived environmental models without taking into account the specific configuration of the new landscape can lead to false predictions, sometimes with catastrophic results. Human groups have to reach a balance between the unknown consequences of using their prior knowledge and the investment of critical time to acquire new learning (Meltzer, 2003). For this reason, populations prefer "legible" landscapes (Golledge, 2003), in which processing demands are low and generalised knowledge can be successfully applied. Environmental homogeneity is an important factor in this context (Gamble, 1994): more continuous, rapid dispersal can be expected in macro-environmental zones with a similar set of resources (Beaton, 1991; see also Osorio et al., 2017). Initial populations are better prepared to track "gross habitats" (Meltzer, 2004) which become more spatially specific and differentiated only with increased residence time, even if this inhibits the ability to continue dispersing (Wren and Costopoulos, 2015).

At least three components of the landscape have to be learnt: regimes, routes and resources (Meltzer, 2003). Track regimes require users to recognize and predict a broad variety of climate patterns such as temperature oscillation, precipitation, annual snowfall, etc. Knowledge of regimes normally extends from the specific to the general. Since climate is experienced over a few years within the life of an individual, intra-annual variation and even some decadal fluctuations can be learned easily, unlike those occurring in centennial-millennial cycles (Meltzer, 2003). Learning and tracing routes involves a different cognitive process known as wayfinding in which individuals identify and interpret parametters of the landscape as they move through it, using memory and the spatial knowledge base to go from one place to another (Golledge, 2003). In complex environments, more time is invested in the creation of rigorous cognitive maps in order to avoid the risk of getting lost (Golledge, 1999). Initially, settlement systems would consist of a few stable points (landmarks) using prominent topographic and geographic features as references and connected by safe pathways such as natural corridors. Over time, they evolve into more complex layout-based systems with a greater number of landmarks and links (organized according to their frequency and purpose of use) in which nested areas are differentiated around them (Golledge 2003). In this case, the associated

knowledge goes from the general (or "extensive") to the specific. Learning about resources depends on their reliability, availability and predictability (Rockman, 2003). In the case of fixed, permanent resources, such as sources of lithic raw materials or water, knowledge is less transferrable since it depends on local factors. Impermanent resources on the other hand, require the development of limitational knowledge about their characteristics, boundaries and costs (Rockman, 2009). However, it can be transferred over larger regions, especially in the case of bigger, more mobile animals (Kelly and Todd, 1988; Gamble, 1995; Kelly, 1995).

2.1 Scouting, hunters and trackers

Hunter-gatherer societies use different strategies for learning the landscape. Scouting is a common activity for acquiring information through direct observation during rapid exploratory forays (Meltzer, 2004). However, exploring is rarely the sole objective of these expeditions and scouting is usually embedded in hunting parties (Roebroeks, 2003). Tracking and stalking animals, or simply following their trails allows routes and wildlife ranges to be mapped and new patches of resources incorporated into cognitive maps (Nieves and Stoffle, 2003). Hunting is a complex cognitive task in itself, that entails high information-processing requirements and real-time resolution of spatial problems in a coordinated and collective way (Tolan-Smith, 2003). During their forays, hunters depend primarily on a route-based knowledge and several wayfinding strategies such as “piloting” (navigate between distant landmarks) and homing via “path integration” (dead reckoning) (Golledge, 2000). For this reason, scouting trips are commonly conducted by expert hunters and trackers with advanced wayfinding skills, motivated not only by the acquisition of resources but also by the chance to gain social prestige (Fiedel and Anthony, 2003).

In a state of partial uncertainty, scouting provides additional information to support the decision-making on what to do next (Jochim, 1976). Large-scale migration generally involves a previous scouting phase (Hegmon and Fisher, 1991), however scouting can also be a constant activity during the annual cycle to incorporate fall-back or full-use areas (Nieves and Stoffle, 2003), or when game is scarce (Binford, 2001:454). Information about a large area or from focused events can be captured quickly and at low cost, however, its circulation is restricted to the local or inter-band network (Fitzhugh et al., 2011). These expeditions are composed of one or a few individuals who quickly move out of their home range in search of prey. Logistical mobility is probably the best option since it imposes fewer limitations on movement and greater risks can be taken (Kelly, 2003). Scouting trips can cover long distances and in some cases last for several months (Binford, 2001). During the course of the expedition, short-term specialized task camps are used for one or a few nights, principally for overnight stays, fire maintenance, tool replacement and repair, processing of captured prey animals and discrete consumption (Lovis, 2005). Within their cognitive maps, scouts and hunters can manage a wide repertoire of potential refuges in case of need, such as caves and shelters (Walthall, 1998).

2.2. Information pooling and hunter-gatherers’ networks

During their face-to-face interactions, individuals establish and strengthen bonds with other individuals and so expand their personal networks; in this way, knowledge of the landscape available beyond their local niche can be transmitted and updated. Foragers invest time and resources in networking, often for the purpose of acquiring relevant information of the environment – even of distant, unknown areas beyond their direct experience (Silberbauer, 1981). Regular, multi-band aggregations for periodic ceremonies, trading fairs, collective tasks, etc. are good opportunities for members of neighbouring groups to update and exchange knowledge (Fitzhugh et al., 2011). They allow information to be acquired in large volumes and at a low cost by indirect or “down-the-line” transmission. However, as the distance increases, the information is more prone to distortion, and accurate information of remote areas is more selective as well as costlier; it requires the investment of a greater amount of energy and material resources in order to maintain stable, frequent interactions (Fitzhugh et al., 2011). In this context also, logistical mobility is an important strategy for supporting visits between reciprocal partners, trade expeditions, pilgrimages or extended journeys. Given the greater distances, these interactions generally involve the exchange gift-giving of non-utilitarian goods in order to ensure the strength of social ties (Whallon, 2006).

In forager societies, mechanisms for circulating information are mainly verbal and co-presential and this implies a greater risk of loss or distortion. In the absence of material supports, the only way of storing knowledge is in individual memory (Whallon, 2011). Maintaining large, cohesive networks allows the environmental information acquired through direct observation (after scouting, for example) and individual experience to be pooled and transferred to larger social units (Tolan-Smith, 2003). In this way, knowledge of the landscape can be trasmitted not only in space, but also in time through intergenerational exchange, allowing events of greater amplitude to be tracked, with an interannual-decadal or even centennial frequency (Fitzhugh et al., 2011).

3. Regional Settings

3.1. The current landscape

The structure of the highlands of the south-central Atacama (Fig. 2) is modelled by two major morphostructural units: the Domeyko precordillera to the west, and the Andes Mountains, rising to above 6000 masl, in the east (Fig. 3A). This abrupt vertical gradient determines a strong contrast in precipitation regimes: while the mean annual precipitation (MAP) is close to 300 mm at 5000 masl, below 2300 masl it can decline rapidly to less than 1 mm (Houston, 2006a). Currently, more than 85% of the annual rainfall occurs during the southern summer (December to March), when moist air masses are transported from the Amazon, triggering intermittent convection storms (Garreaud et al., 2003). In contrast, winter rainfall is related to the Pacific moisture which penetrates from south-central Chile, often in the form of snowstorms (Vuille and Ammann, 1997). On an inter-annual time-scale, rainfall regimes are modulated by El Niño Southern Oscillation (ENSO) (Houston, 2006b). During La Niña, summer rainfall tends to increase producing massive flooding; conversely, extreme winter precipitation and summer droughts are associated with El Niño phase. While precipitation regimes can present significant irregularity and unpredictability (Romero et al., 2013), groundwater recharge represents a more reliable fresh water source (Houston et al., 2016b). The saline lakes and salt flats located on basin floors are fed by numerous inflows which discharge their water into the hydrological base in the form of diffuse seepages, flowing springs, perennial rivers and aquifers (Risacher et al., 2003).

Compared with the northern Atacama, the flora becomes poorer further south due to the greater aridity. At this latitude (21-22ºS), the maximum inland penetration of the desert reaches 2700 masl (Arroyo et al., 1988), above which an herbaceous and shrubby zonal vegetation develops, distributed in altitude-conditioned vegetation belts. At least four such belts can be recognized in the study area (Fig. 3B): (1) pre-puna (2700-3100 masl), characterized by semi-desert deciduous shrubs, succulents and cacti; (2) puna (3100-3750/3900 masl), scrub dominated by shrubs or *tolas*; (3) high puna (3750/3900-4400 masl), composed of shrubby steppe (caespitose perennial grasses or “pajonal”); and (4) the subniveal belt (>4400 masl) which marks the upper limit of the vegetation (Aldunate et al., 1981; Villagrán et al., 1981, 1998).

In contrast, the azonal vegetation maintains permanent cover throughout the year. Currently, some 264 active wetlands, marshes and bogs have been identified in the region (DGA, 2004). They are dominated mostly by species of reeds, grasses and cushion plants, which grow round the margins of lakes and salt flats and on the banks of canyons (Villagrán and Castro, 1997). Even in the arid lowland desert, azonal vegetation grows in scattered oases, supporting low-elevation wetlands and trees such as tamarugos (*Prosopis tamarugo*), algarrobos (*Prosopis alba*) and chañar (*Geoffroea decorticans*) (Villagrán et al., 1998). Azonal formations are vital for fauna. The majority of animals are residents and reproduce in the region, especially less mobile species; these include micro mammals such as chinchilla (*Chinchilla brevicaudata*) and vizcacha (*Lagidium viscacia*). Larger species such as wild camelids (*Vicugna vicugna* and *Lama guanicoe*), canids (*Lycalopex culpaeus* and *Pseudalopex griseus*) and felines (*Lynchailurus colocolo*, *Oreailurus jacobitus*, *Puma concolor*) can travel great distances and reproduce outside the area.

3.2. Paleoenvironment and climatic changes

The Atacama is one of the oldest and driest deserts on earth, however the paleoenvironmental record shows a dynamic past marked by pulses of higher humidity. After the Last Glacier Maximum (LGM), a climate change event known as the Central Atacama Pluvial Event (CAPE) increased summer precipitations in the Andes region in two phases (Latorre et al., 2006; Quade et al., 2008; Placzek et al., 2009). The first is known as CAPE I (17.5-14.2 ka BP) and affected mainly the Central Andes; in contrast, CAPE II (13.8-9.7 ka BP) was much more intense and prolonged in the south-central Atacama. During this phase, the rainfall was 3 to 5 times higher than today, reaching a mean annual precipitation of 400-500 mm (Grosjean et al., 2001). Some lakes of the high puna grew to more than six times their present area. Water bodies covered nearly 8% of the area as opposed to barely 0.9% as now (Kull and Grosjean, 1998). Particularly illustrative have been the studies in Miscanti (4140 masl) (Fig. 4A) which show higher lacustrine levels between 14 and 9.5 ka BP, disrupted by a short arid interval around 11.6 ka BP (Grosjean et al., 2001).

The higher hydrological recharge also raised the levels of subterranean water tables in pre-Andean basins, forming large wetlands, marshes and bogs (Rech et al., 2002; Quade et al., 2008; Sáez et al., 2016) (Fig. 4B, Fig. 4C). As result, the diversity and abundance of plant species increased considerably. Steppe grasses currently relegated to the high puna descended, while northern plant species extended at least 50 km south of their modern ranges (Betancourt et al., 2000; Latorre et al., 2002, 2003; Maldonado et al., 2005). Some studies indicate that the primary productivity was 10 to 20 times greater than at present, allowing the development of 50 to 80% plant cover, compared to <5% now (Latorre et al., 2002) (Fig. 4D). Even on the desert floor, peats and organic paleosoils with gastropods and layers of diatomites have been dated to around 11.2-10.5 ka BP (2200 masl), showing that the lacustrine environment and marshes of the Atacama salt-flat expanded 1200 metres beyond its current border (Núñez et al., 2005). Similar evidence has been found in saline cores extracted from the Atacama salt flat, revealing an Early Holocene wet phase from 11.4 to 10.2 Ka (Bobst et al., 2001). Humid conditions extended even into the Central Valley, as recently indicated by the analysis of organic and saline sediments in Pampa del Tamarugal (900-1000 masl) (Nester et al., 2007; Gayó et al., 2012) and the lowlands of the Antofagasta region (900-1600 masl) (Pfeiffer et al., 2018).

However, during the second part of the Early Holocene, the aridity increased drastically in the South-Central Atacama (Sáez et al., 2016), and this situation became more severe during the Middle Holocene (Grosjean et al., 2001, 2003). Although the impact of this climate change on human dispersal is still unknown, it triggered a drastic reconfiguration of hydric and biotic resources. Some areas were more severely affected than others. In the high puna, the lakes contracted and most of them dried out (Messerli et al., 1993; Grosjean et al., 2001), while wetlands on the pre-Andean floor collapsed permanently (Latorre et al., 2003; Grosjean et al., 2005; Quade et al., 2008).

4. Sites and stratigraphic contexts

The archaeological sites studied in this work (Fig. 5) are located in the highlands (2000-4500 masl) of the south-central Atacama (21-25°S), covering a temporal range spanning from 13,000 to 9000 cal. BP (Table 1). In the case of the sites in caves and rock shelters, no important disturbance problems are recorded (Núñez et al., 2005). Conversely, differential vertical selection processes have occurred in the open-air sites; this means that archaeological remains have been redistributed in the vertical axis according to their size, shape and weight, generating a mainly superficial record with low stratigraphic potential.

5. Climate regimes

Climate regimes are among the most important component of the environment that must be learned in arid landscapes, since meteorology controls the availability and timing of water recharge. The spatial distribution of the sites and the radiocarbon dates obtained provide a first approach to this problem. As Figure 6 shows, during the Late Pleistocene there was continuous occupation from 13,000-11,000 cal BP, restricted to the pre-puna belt (2600-3200 masl) where no temporal gaps are observed (Fig. 6). The wetlands, bogs and marshes formed during CAPE

II offered productive habitats in the pre-puna floor between the high puna and the lowland desert, which were subject to less seasonality and minor resource incongruence. Given the longitudinal climate gradient, the spatial distribution of the sites would have allowed to access to a wide variety of complementary environments on the same altitude floor.

Intra-annual variability must certainly have been learned quickly, as well as those interannual-decadal events which can be experienced within a lifetime. On the other hand, periodic events occurring on a centennial scale and longer-term changes were more difficult to predict and anticipate. This may be the case of the prolonged drought episodes that caused the abrupt fall in lake levels in Miscanti Lake around 11,600 cal BP (Grosjean et al., 2001), and the oxidized horizons and eroded surfaces in SPN-1 (Grosjean et al., 2005). Although this arid event has not been explored in depth, it occurs contemporaneously to a notable gap in the archaeological signal around 11,500 cal BP. Only after 10,700 cal BP, some sites are reoccupied while others seem to have been re-sited close to more stable water resources – like SPN-19/20 located in the alluvial fan of the Frio River (Cartajena et al., 2014), and TU-109/E-IV placed in the upper course of the Tulán River. In situations where the group is unable to predict the length, amplitude and periodicity of climate changes, temporary abandonment of some sites and territorial change could be a good strategy to evade resource failure and water scarcity. It is precisely during the archaeological gap that incursions into low-elevation wetland areas of the desert level are observed in Tam-2/4a and TU-68 (Núñez et al., 2005).

As the arid conditions of the second part of the Early Holocene settled, the pre-puna was definitively abandoned about 9700 cal BP after the paleo-wetlands dried up and became phreatic beaches. Almost simultaneously, the previous occupations of the desert lowlands gave way to the large multi-seasonal camps at TA-1, and others on a smaller scale such as at TU-67. Stable access to the high puna is noticed in sites such as AC-1, TUY-1, and SM-4, and several other sites that to date have not been dated. Even today, occupation of this area is restricted to summer when the snow melts and grasses are more productive because of the summer rains. It could be argued that greater knowledge of intra-annual variability allowed groups to deploy a settlement/mobility system based on semi-permanent settlement of the lowlands combined with seasonal access to the high puna. This would allow efficient exploitation of resources according to their seasonal availability in the landscape. In the long term, greater systemic resilience was achieved and groups coped better with climate fluctuations and unpredictable events.

6. Routes and pathways networks

Learning routes means internalizing at least two aspects: the location of places and the structure of the paths that connect them. Sites can be divided into two main location patterns (Fig. 7). One group consists of open-air sites placed on fluvial terraces and phreatic beaches around the borders of salt-flats and saline lakes (Table 2); their record consists mainly of surface scatters on modern sedimentary units (Qa, PPl1c), and aeolic litho-stratigraphic units (Qe) related with alluvial activity dating from the Pleistocene-Holocene (Fig. 8). The advantage of open-air camps is that they can be sited directly in the area where resources are available, supporting a practically unlimited number of people; however, this requires the repeated manufacture of stone structures, or use of tents, which have to be dismantled, transported and set up again in every residential move. The second group consists of sites located in rock-shelters and caves on the sides of ravines in the western Andes and the precordillera range (Table 2). Locations of this type are available mostly in volcanic outcrops or under ignimbrite beds exposed by lateral erosion in the walls of ravines (Fig. 8). While caves and rockshelters offer protected environments that require less work investment, their use is severely limited by their availability in the landscape, which may mean less than optimal location with respect to resources, and the often small size of their interior space. By making use of a combination of rockshelters and open-air sites, a balanced relation between mobility costs and distance to resources can be achieved.

Pathway networks (the material expression of routes) are harder to study, mainly because they are not conserved in the archaeological record. However, by using least-cost path analysis we can at least estimate the potential routes that impose the fewest limitations on human displacement. This is not a minor issue in a steep relief like that of the Andes, where slope and elevation are critical factors for mobility. During the Late Pleistocene, the distribution of

settlements not only enabled access to a wide diversity of environments, but great altitudinal contrasts were avoided by maintaining sites within a single ecological floor. Furthermore, the pre-puna facilitated wayfinding thanks to the presence of prominent topographical markers in the Andes and Domeyko ranges, which run parallel in a north-south direction. The preandean basins formed between the two ranges constituted a natural corridor at intermediate altitudes, where the effects of hypoxia are still slight. Certainly, the bottoms of the basins and borders of salt flats were the lowest-cost, safest routes. On the other hand, walking times between the sites not to exceed one day's march (8hrs) (Fig. 7). Such itineraries would have allowed the deployment of short-term pathways used during everyday activities whose accessibility would not have been seriously affected by seasonal or climatic factors. This does not mean that the sites were synchronous, but it allows us to estimate the distances between different relatively contemporaneous localities.

During the Early Holocene, a vertical mobility pattern connected the lowlands and the high puna along an east-west axis of about 70 km, with a drastic altitude range from 2000 to 4500 masl. Of course, this posed new and contrasting challenges to route-learning: on the one hand, the flat, featureless relief of the arid desert lowlands made wayfinding more difficult and riskier; on the other, the steep landscape and hypoxia considerably limited displacement within the high puna. Above 3800 masl, decisions on when and where go up there are critical since even today adverse weather conditions impede access for most of the year. Ravines and canyons seem to have been the safest, optimal routes, and not only for the lower slopes; their rocky walls and caves could be used as refuges or as outposts to monitor the weather, waiting for the right moment De Souza, 2004:39).

Cognitive maps included a large number of landmarks and links between them, and areas which were organized according to their seasonal availability throughout the annual-round. Moving in such a complex layout system surely required a deeper route-based knowledge, the more so in a changing environment. The large number of Early Archaic sites in the high puna could be the result of successive adjustments in settlement location as lakes dried out (Fig. 7). For instance, SM-4 is both the latest site chronologically and that located closest to the edge of the lake at its current level (Núñez et al., 2002); the same is true of the large number of sites in the Tambillo oasis. Since the camps were mainly open-air sites, greater flexibility is apparent in their placement selection. In this scenario, paths and routes were more hierarchically organised. Some were used daily within an ecological floor, others were used seasonally during the displacement of the group between adjacent or distant ecological floors. Some must also have allowed travel to remote areas outside the region during intermittent journeys, requiring the group to cross challenging barriers such as the Andes Mountains and the arid plains of the central valley.

7. Resources

7.1. Fresh water

Fresh water is a fixed resource but its availability can be strongly affected throughout the annual cycle. Furthermore, water quality and composition can vary widely, even between sources located a few metres away from one another. Most have a high salinity (according to the amount of Total Dissolved Solids, Fig. 8) or a high content of elements such as arsenic and boron (Risacher et al., 1998). Not only must the location of waterholes be learnt but also their characteristics and limitations for human consumption. Not surprisingly, almost all the sites are located near the few good quality water sources. These are mainly flowing springs, probably because rivers and lakes have high salinity due to the evaporation rate.

While Late Pleistocene sites are generally located in currently dry environments, there are active springs within a few kilometres (i.e. Fig. 8A and B). In the case of Imilac and Punta Negra basin, sites are placed at the border of salt-flats close to high quality groundwater sources. However, the modern water table is at a depth of 70 meters (Risacher et al., 1998) and therefore inaccessible (Fig. 8C, 8D, 8E and 8F). It can be established that Late Pleistocene settlements were occupied only when groundwater levels were high; once the fresh water sources dried up or became inaccessible, the sites were abandoned. Conversely, Early

Holocene sites, especially from the second part of the period, are located directly at currently active water sources. This may be due to two reasons: the greater water availability stress to which the settlements were subject and the reconfiguration of hydrological cycles as aridity increased. The high puna sites are located directly at good quality springs exposed when the lakes contracted to their current size (Fig. 8 G.H and I), while the occupation of the lowlands coincides with the formation of groundwaters in the Atacama salt-flat (Fritz et al., 1979) (Fig. 8J and 8K).

7.2. Rocks

Knowledge of lithic resources is less transferrable since it depends on local factors such as geology and geomorphology. The resources which were easiest to identify in the landscape were quickly acquired once their location became known (Ford, 2011). Coarse-grained rocks such as basalts, andesites and felsites can be found in large, highly-visible primary formations related to mountainous geoforms, which also explains their high frequency in assemblages (Fig. 9). Such is the case of the extensive outcrops of tuff in Tulán Cerros (Fig. 8K) and aphanitic basalt in Morro Punta Negra (Fig. 8F) (Loyola et al., 2017). Siliceous rocks show a greater frequency, even though they are not always available locally; moreover, they are more difficult to identify. In this case, some patterns in the lithic landscape could be used as “schemata” (Golledge, 2003). In the highlands, white siliceous rocks are usually found in smaller residual sources included in secondary sedimentary deposits. Something similar occurs with chalcedonic breccias, which have been recorded in discrete hydrothermal craters on the northern edges of Imilac Salar (Fig. 8C). A few kilometres away, there was an extensive phreatic beach where small nodules of white flint of excellent quality have been found. An exception is the Talabre source, where several workshops has been documented associated with large outcrops of siliceous rocks (De Souza, 2010).

In the Andes, obsidian is found in small domes and lava-domes associated with large calderas and stratovolcanoes (Yacobaccio et al., 2004). A large number of the obsidians in the assemblages come from the local source of Pelún, north of the Salar de Atacama (Seleenfreund et al., 2010), demonstrating early knowledge of this source. Black obsidian, on the other hand, was acquired mostly from sources in the Zapaleri-Tara system located in the high puna, where it appears in the form of scattered small nodules (Fig. 8H); sites such as AC-1 ensured supply to lower floors, although procurement was seasonally restricted. Extra-regional obsidians are also recorded, sourced from sites in northwestern Argentina such as Tocomar and Quiron (Escola et al., 2016). Towards the Early Holocene, a substantial increase of transported obsidian is observed in the assemblages (Fig. 9), particularly extra-regional varieties. At the same time, some presumably local sources, such as grey obsidian which had not previously been exploited, achieve a wide spatial diffusion, suggesting deeper knowledge of the local lithic landscape.

7.3. Scanning plants

Although the lower biodiversity of plant species imposed a lower learning demand, the significant endemism of the native flora (Squeo et al., 1998) could disable the application of prior knowledge. The ecological floor structure defines specific altitude distributions for each species, whose growth habits, nutritional potential, limitations, functionality and availability throughout the annual cycle must be learnt. A general classification of the native flora, based on taxonomic and physiognomic similarities with already known plant species (of the same family, for example) could have been a good way to learn local plants (Villagrán et al., 1998). The case of azonal vegetation could be different. Due to the biogeographical expansion during the Late Pleistocene, azonal plant formations show a peculiar homogeneity throughout the Central and Desert Andes (Villagrán and Castro, 1997), constituting a more reliable and better-known source of plant resources. Unlike zonal vegetation, which follows seasonal growth timings, bogs and marshes maintain continuous, dense cover throughout the year, even during the cold season. Besides being the main source of forage for camelids, this formation is also the most important source of edible and medicinal plants, firewood, pigments, plant fibres and other resources such as eggs (Villagrán and Castro, 1997).

In the Late Pleistocene and Early Holocene sites, there is evidence of active paleowetlands, contemporaneous with human occupations, which disappeared with the increasing aridity. For instance, silt deposits have been recorded in Imilac and Punta Negra which contain epiphytic diatomites and Cyperaceae, as well as root marks, intercalated with peat layers (Grosjean et al., 2005), sponge spicules, algal testae and gastropods (Succineidae) (Quade et al., 2008). In the Tuina sites, steppe grasses, abundant summer annuals and tolar shrubs have been reported between 11,700 and 9600 cal BP (Latorre et al., 2003) (Fig. 8A). At the SL-1 site, the cultural material is contained in sand layers with abundant organic content and plant remains (Núñez et al., 2005) (Fig. 8B). Evidence of bulbs or succulent roots that grow during the rainy season was also recovered (Núñez et al., 2005).

Conversely, Early Holocene sites are directly associated with marshes and wetlands after the reconfiguration of water resources. In the case of high puna sites, there are bogs around the springs located at lake margins (Fig. 8G, H and I). The Tulán River sites are located a few hundred metres away from the Tchulin Marshes (Fig. 8K) (Betancourt et al., 2000; Latorre et al., 2002). In TA-1, plant macrofossils and gastropods dated to 11,190-10,580 cal. BP (Geyh et al., 1999; Núñez et al., 2005) have been collected from an area one kilometre to the east of the current shoreline, where there are now marshes and introduced Tamarugo forests (Fig. 8J). Arboreal resources seem to have acquired special importance at the end of the Holocene based on the appearance of profuse grinding technologies in TA-1 and chañar seeds recovered in TU-67(E-VII) (Núñez et al., 2002). The fruits of these trees grow during the summer, but if ground into flour they can be stored for a long time. It is difficult to specify whether these arboreal resources were known previously. In any case, learning how to process them would require some degree of observation and experimentation, which usually happens during periods of environmental change (Meltzer, 2003: 232).

7.4. Animal-tracking

According to the available evidence, subsistence was based on a broad-spectrum generalist diet. The consumption of animals focused almost exclusively on modern fauna species with emphasis on wild camelids (*Vicugna vicugna* and *Lama guanicoe*), even when extinct mega fauna was available (Fig. 10) (Cartajena, 2003; Cartajena et al., 2006). Certainly, the tracking and capture of wild camelids required knowledge of their feeding behaviours, social organization, demography, seasonality, reproductive habits and migration routes. In an environment of high spatio-temporal incongruence of forage plants and heavy snowfalls, guanacos can develop seasonal migratory and reproductive patterns. It has been observed in several areas of the Andes that guanacos disperse into smaller units on lower floors during the winter, but as soon as the snow melts they return to the highlands for the summer breeding season (Franklin, 1982; Cartajena, 2003; Puig et al., 2011). Vicuñas on the other hand have more sedentary and territorial habits, limited to the upper floors.

Camelids can be found in most of the Andes Range, even in Patagonia in the case of the guanaco. They constituted one of the main resources in the diet of the early hunter-gatherer groups of the Andes, suggesting that their behaviour and ecological cycles were widely known. Unlike micro-fauna, larger, mobile game enables the use of more generalized knowledge, transferrable between distant regions. It was only necessary to acquire minimal limitational information (Rockman, 2003). Furthermore, camelids' migration patterns seem to have been quite different during the Late Pleistocene. The representation of different-aged segments in archaeological assemblages suggests the presence of stable guanaco family groups throughout the year in the intermediate floors (adult males and females, calves, and juveniles born the previous year before they are expelled from the group) (Cartajena, 2003). In the case of vicuñas, it is not possible to state whether they were present or if they were transported from the high puna (Cartajena, 2003; Cartajena et al., 2006). Either way, the greater humidity seems to have supported more stable faunal resources in the intermediate floors which were less affected by seasonality patterns, even attracting extinct species (*Equidae*) and others that no longer inhabit the region such as deer (*Hippocamelus antisensis*) (Cartajena, 2003).

Camelids continued to be the main source of subsistence during the Early Holocene, but some evidence suggests adjustment to more pronounced seasonal distribution and mobility (Cartajena et al., 2006). For instance, the high representation of adult individuals in sites such

as TU-68 and TA-1 point to prolonged occupations during the cold season. On the other hand, at TU-68 there is a remarkable frequency of Andean Doves (*Metriopelia* sp.), which nest in trees or rocky areas between April and August; while the presence of *Phoenicopterus* sp. in TU-67 and the high frequency of vicuñas in TA-1 could be the result of a more stable human access to the upper floors during summer. In addition to birds, other complementary local resources were important – such as vizcacha (*Lagidium viscacia*) which have crepuscular habits and frequent steep rocky areas in ravines of the pre-puna, and fossorial rodents like *Abrocoma* and *Ctenomys fulvus* that live in sandy environments of the desert lowlands.

8. Lithic technology and landscape learning

8.1. Scouting camps and lithic assemblages

For hunter-gatherers, lithic technology is closely linked to mobility, hence the lithic record gives us clues to understand how they learned the landscape as they moved about in it. There is a particular group of assemblages with a very low abundance of artefacts (SL-1, TU-109/T-1, Tam-2/4a, TUI-1 and SPN-6) (Fig. 9). This appears to be not merely a sampling issue, since an obvious technological pattern stands out: these assemblages are mainly composed of small flakes detached during the maintenance and use of retouched tools. Such tools were often made on distant raw materials such as obsidian or siliceous rocks, and were apparently already finished when they entered the sites. After occupation ended, they were transported back by their owners as part of their personal equipment. This would explain why the tools themselves are mostly absent, while their by-products are present. Just a few projectile points, knives and side-scrapers have been recovered in some of these sites, either broken or quite exhausted. Immediately available coarse-grain rocks from the area surrounding the settlement were used as sharp-edged tools. Bone remains and hearths are also recurrently found together with the lithic artefacts. Undoubtedly, hunting and primary processing of camelids were the central activities, although there was also some *in situ* consumption.

Everything seems to indicate that these small occupations were used only briefly as specialised-task sites within a wider settlement system; certainly, long-distance logistical mobility would fit quite well with this archaeological signature. Several of these sites also have very early dates that long precede the more stable residential camps in each locality. It may therefore be assumed that occupations of this kind allowed hunters from remote areas to explore and scout the landscape as part of hunting trips. In Tam-2/4a, for instance, just a few retouching flakes of an extra-local obsidian were recovered in a peat layer, constituting the first incursion so far known into the desert lowlands. The same applies to TU-109/T-1, where the oldest date for Tulán ravine and one of the earliest for the area was recorded. Despite their excellent quality and proximity, local raw materials such as Tulán tuff and grey obsidian are completely absent. In fact, the presence of green felsite could indicate a link with the Tuina area, more than 130 kilometres away.

The other sites show a similar pattern and early dates, but with more redundant use, or for longer periods. SPN-6 is an open-air hunting stop used at least twice; a few exhausted tools, an unmodified agate and flakes employed for primary processing and consumption of camelids were discarded after use around an exposed hearth (Fig. 11A and B). SL-1 was also reoccupied intermittently, presenting very low abundance throughout the entire sequence and interrupted by long gaps when it was abandoned. Conversely, in TUI-1 there are no visible periods of abandonment and the abundance and diversity of artefacts (some of them made on local obsidian) indicate greater occupational intensity. TU-109/E-IV is a later occupation in the eastern sector of the TU-109 rock shelter; it allows us to appreciate how the local landscape had been learned by then: beside the greater abundance and diversity of artefacts, local Tulan tuff and grey obsidian clearly predominate (Fig. 9).

8.2. Lithics, information and networking

Many of the social interactions in which lithic artefacts were involved occurred in the base camps like TA-1, SI-7, SPN-1 and SPN-20. The occupational redundancy and frequency of such interactions led to high discard rates, forming dense lithic assemblages scattered over large areas. Immediately available rocks procured during daily foraging activities were most

often used; they were transported to the camps in bulk in the form of cores or blanks. Thus, almost all the phases of the operational chains were carried out, from production through use to final discarding. The diversity of the toolkits also indicates that multiple tasks were carried out, linked to primary and secondary processing of animals, plants and mineral resources. In TUI-5, AC-1, SM-4, TUY-1, TU-67 and TU-68, although they are also medium-size occupations, the diversity and classes of tool (and other complementary evidence) suggest the performance of more specialized tasks.

A plausible scenario to explain size and functional discrepancies is the existence of seasonal fission/fusion strategies. Periodic macro-band aggregations would be a very auspicious context for acquiring and transmitting information. A certain homogeneity in lithic technology suggests sharing of technological knowledge. The clear predominance of the triangular, non-stemmed "Tuina" points, recorded in several sites in the south-central Andes, could be a good example (Fig. 12A). Other projectile points such as fishtail (Fig. 12C), although they are an unusual find in the Atacama Desert, are distributed throughout the whole of South America. Other shared patterns are also reflected in production modes, for instance the selection of large angular flakes obtained from cores of low technical investment (Fig. 11J) as tool blanks, which are then subjected to high edge complementarity and retouch intensity (Fig. 11A, C and F). Likewise, the use of cores on flakes and specific techniques for recycling exhausted tools, such as inverse retouching or the production of intentional fractures.

Towards the Early Holocene, important changes occur in lithic technology. In the first place, the assemblages show greater tool diversity and specialization. Retouched tools present smaller sizes as well as lower edge complementarity (Fig. 11 G, D, H and I). Some types not previously recorded, such as drills (Fig. 11E), make up a large part of the assemblages, indicating the importance of other secondary processing activities. The same can be said of the profuse grinding technologies in TA-1 (Fig. 11K) and other sites located on the margins of the Tambillo paleowetland, where extensive grinding and gathering areas have been described. In the case of bifacial operative chains, new projectile point designs are incorporated, some requiring greater technical investment. The stemmed, barbed "Punta Negra" (Fig. 12B), although mostly superficial, are frequently found in sites dated around the first part of the Early Holocene, while other designs are more characteristic of the second part, e.g. pentagonal (Fig. 12G), tetragonal "San Martin" (Fig. 12D) and the triangular, non-stemmed "Tambillo" (Fig. 12F). A similar, and contemporary, pattern of projectile point diversification has been observed in northwestern Argentina, attributed to changes in hunting organization. It has been suggested that during the Pleistocene-Holocene transition long-distance individual hunting predominated, based on the use of weapons thrown with spear-throwers (*atlatl*), but that by the end of the Early Holocene a collective hunting strategy had been developed using short-range, hand-thrown spears (Aschero and Martinez, 2001). Collective hunts have been proposed in parallel for sites in the high puna (Núñez et al., 2005). The frequent presence of projectile points, knives and scrapers indicates that there was a strong emphasis on hunting activities and animal processing in these large, dense seasonal camps.

In a context of greater territorial circumscription, individuals spend more time away from the camps to acquire high-ranked resources. The inter-band aggregations outside the residential area, either for collective hunting in the high puna or plant-gathering in lowland oases, would encourage cooperation and the transmission of knowledge from the elders to the young. Lithic raw material sources are unlikely to have been an exception. Since apprentices require large amounts of raw material, learning takes place sporadically in those places (Piegot, 1990). Some bifacial operative chains, such as Punta Negra points, required learning of more elaborate technical skills through active tutoring, for example. The case of SM-4 is quite illustrative. A large quantity of tetragonal projectile points were produced on immediately available basalt; however, the majority were rejected due to knapping accidents or morpho-functional deviations, while others were manufacturated with great skill. Such differences in levels of technical competence would be expected in an apprenticeship context.

The technological similarities of our study area with northwestern Argentina have led to the proposal of a "Tuina-Inca Cave" cultural complex (Aschero and Podestá, 1986). The identification of obsidian from the eastern slope of the Andes proves the existence of early contacts between the two areas, over distances exceeding 200 km, meaning that mountain

passes over the Andes were already known from an early period. Judging by the remarkable increase in extra-local obsidian transport, social contacts were more stable and frequent by the end of the Early Holocene. By intensifying their long-distance ties, groups also increased their access to landscape information to cope with environmental fluctuations. Given the great distances involved and the steep relief of the Andes, logistic mobility would be a more effective strategy to achieve such face-to-face interactions, for instance in the form of social visits between reciprocal partners or small expeditions with the objective (among many others) of acquiring information. The circulation of obsidian, mainly in the form of projectile points (Table 3), is quite suggestive. It has been argued for later periods that obsidians were used as tokens or exchange goods (Escola et al., 2016). This could explain the importance acquired by bifacial knapping at the end of the Early Holocene, and not only in terms of design diversity. The percentage of bifacial products and by-products in the assemblages is striking, often involving more elaborate thinning and final shaping procedures.

9. Discussion

9.1 A gradual process?

The evidence to date shows that Late Pleistocene occupations (13,000 to 11,500 cal BP) were limited exclusively to the pre-puna floor (2700-3200 masl), indicating that hunter-gatherers selected the more productive and legible habitats available on this floor in the initial stages of dispersion into the area. The high puna and desert lowlands were only included in the settlement process later, at the end of the Early Holocene. However, we cannot infer a positive linear relationship between landscape learning on the one hand and diversity of inhabited environments on the other. The synchrony between prolonged droughts and different abandonment and relocation events suggests a more complex scenario, perhaps better described as a process of learning and experimentation with local resources. After the abandonment of the pre-puna, this process culminated in complementary land-use of the highlands and lowlands – paradoxically, the two areas considered more risky. This structured pattern of seasonal mobility did not simply allow groups to exploit efficiently the environmental variations of the annual cycle; combined with more specialized technologies, it was a key strategy for dealing with climate change.

However, if we compare this colonization process with that in neighbouring areas, we detect important differences in timing. In the northern Atacama (Osorio et al., 2017), northwestern Argentina (Yacobaccio et al., 2017) and the Bolivian altiplano (Capriles and Albarracin-Jordan, 2013), occupation of the high puna occurred substantially earlier, and the same appears to be true of the lowlands, based on information from the sites in the Pampa del Tamarugal (Latorre et al., 2013). Why is there no record of previous occupations in the south-central Atacama outside the prepuna floor? There are two possibilities: either these areas were indeed colonized later due to adverse conditions for human subsistence, or there are problems of visibility and conservation of the archaeological record. According to Núñez et al. (2002), the earliest occupations located on the margins of high altitude lakes before they reached their maximum dimensions may have been buried by sediments. The low sedimentation rate and wind erosion represent recurrent problems in these archaeological contexts. Undoubtedly, early incursions could have taken place, but they are difficult to detect in a time-averaged, superficial archaeological record. A large number of human occupations attributed to the Early Archaic have still not been dated.

Although the peopling process could be “gradual” in terms of timing and the diversity of inhabited environments, evidence suggests that the hunter-gatherer groups maintained a way of life already adapted to the salt puna (Núñez and Santoro, 1989; Núñez et al., 2002; Aschero, 2010). At least, there is no evidence to support peopling from the coast – on the contrary, early human dispersion was facilitated by transferable prior knowledge, based on the hunting of camelids and the collection of plants in azonal formations. In their search for richer resources, these groups may have followed dispersion routes through the Andes, tracking megapatches which were more homogeneous than current biomes (Cartajena et al., 2014, Osorio et al., 2017, Loyola et al., 2018).

9.2 Learning to move, moving to learn

During human dispersal into an empty land mass, mobility is practically unfettered; however, arid landscapes pose a special problem for human settlement due to water scarcity. The location patterns of residential camps indicate that placement selection was strongly conditioned by the distribution, availability and quality of fresh water, even during wet climatic conditions. Like many hunter-gatherer groups in desert environments, the first human groups could have followed a "tethered" foraging pattern (Kelly, 2013:93) in which knowledge about the location of the better quality waterholes and recharge cycles was critical for programming mobility (Gould, 1991; Marlowe, 2010; Hiscock and Ebert, 2011). However, the contribution of plant as buffer water reserves should not be ruled out (Silberbauer, 1981). The remains of roots and tubers found in SL-1 could be evidence of such strategy.

Several authors have proposed the existence of high residential mobility during the early peopling of the Atacama puna (Núñez et al., 2002; Aschero, 2010; Yacobaccio and Morales, 2013). Indeed, the abandonment and relocation events recorded in the study sites associated with prolonged droughts could be the result of high residential mobility and more flexible territorial patterns. Foragers in environments with high interannual variations in rainfall employ an “escape and evasion” strategy (Gould, 1991) and constant migrations (Silberbauer, 1981; Cashdan, 1984; Tanaka, 1980). However, settlement patterns and archaeological assemblages point to the existence of complementary logistical mobility. Following Aldenderfer (2006), this could be due to the emergence of more structured foraging patterns in high altitude environments, leading to a decrease in residential movements. Recently, attention has been drawn to the occupation of logistical camps on the high puna floor of neighbouring areas (Capriles et al., 2013; Osorio et al., 2017).

Residential mobility allowed groups to disperse quickly over a large, continuous area; however, it reduced the possibilities of acquiring extensive information on the landscape, especially considering that site location was tied to the distribution of water sources. In a context of incongruous resources or "separate habitats", logistical mobility was a useful solution, also offering better possibilities of capturing environmental information. The interaction of these two mobility strategies had important consequences for the peopling process. When new patches were incorporated into the logistics radius, changes were triggered in residential mobility circuits and new residential camps were attracted.

9.3 Hidden networks and encrypted information

The idea that first human groups of the salt puna were relatively autonomous and maintained highly dispersed social networks has predominated. However, our data suggest that these small, scattered bands managed to sustain social cohesion at the supra-band level and maintain long-distance contacts. Low population density is good way to maintain an adequate immediate return in an environment of low primary productivity, but in the long term it is not demographically viable (Wobst, 1974). If we judge from the differences in the size of the sites and their functional orientations, the occurrence of periodic inter-band aggregations is an eminently possible scenario. A flexible social organization and fluid membership (as part of fusion-fission strategies, for example) would allow to maintain small group sizes while remaining closely interconnected within exogamic networks, ensuring reproductive success and information transmission. However, inferring social aggregations from the archaeological record is always complex (Hoffman, 1994). Other archaeological indicators besides lithic technology will certainly be necessary in future.

How extensive these networks were is just as difficult to establish. Obsidian transport suggests the existence of interaction networks across the 300 km. This is not unusual in small-scale forager bands; indeed, some authors suggest that as the size of the group decreases, the distances travelled to visit neighbouring groups in order to find a mate increases proportionally (MacDonald, 1998). We have suggested the hypothesis that many of these obsidian objects circulated in the form of projectile points as exchange goods, which correlates with hunter-gatherer ethnography (Hiscock and Ebert, 2011). However, there are no reliable archaeological indicators to distinguish indirect procurement through exchange from direct long-distance procurement. Furthermore, what is assumed to be an exchange could in fact be the result of a wide diversity of behaviours and different forms of social interaction (Bruch, 1988), each of

which prints particular signatures in the archaeological record. More detailed technological studies on the circulation patterns of lithic raw materials will provide new perspectives, but for now the existence of generalized technologies with a certain regional homogeneity can give us an idea about the spatial scale of information flows.

Apparently, social contacts were not limited only to the highlands; the coastal area inhabited contemporaneously by the Huentelauquén cultural tradition (Salazar et al., 2017) also seems to have formed part of early interaction networks. Conch shells from the Pacific coast (*Olivia peruviana*, *Concholepas concholepas* and *Argopecten purpuratus*) have been recorded in Early Holocene sites, including TU-67, TU-109/E-IV and TA-1 (Núñez et al., 2005). Other evidence can be found in the similarity of the funerary practices observed in TA-1 (Costa-Junqueira et al., 2001). The intensification and expansion of long-distance interactions at the end of the Early Holocene could be a valid strategy for coping with increased aridity and resource failure, offering a safety net (Whallon, 2006), especially in a context of increasingly contrasting habitats and decreased mobility (Cashdan, 1984; Barnard, 1992). Ultimately, these early interaction networks structured the peopling process itself, facilitating the circulation of resources, people and information. They formed the basis for the emergence of later cultural traditions and social complexity.

10. Conclusions

The highlands of the Atacama Desert probably represent one of the most challenging environments for human colonization, so their peopling was necessarily a gradual process, structured over time as human groups learned this arid landscape. Knowing “what it’s like out there” was a critical and permanent task. Rather than a constant southward movement in the sense of a biogeographic corridor, on a millennial-centennial scale, populations dispersed progressively and selectively at different rhythms and speeds, based on their increasing knowledge of a changing environment. Scouting as part of logistical hunting parties and information-pooling through networking were essential complementary strategies to acquire and transmit information about climate regimes, routes and resources.

Late Pleistocene occupations were initially limited to the pre-puna floor, which offered an intermediate habitat between the extreme conditions of the high puna and the desert lowlands. On this altitude floor, the more humid climate generated productive micro-environments with less acute resource incongruence and less extreme seasonality. The greater legibility and lower information demand encouraged rapid dispersal from other neighbouring eco-regions with a similar ecological structure. Generic prior knowledge based on camelid hunting and plant collection in azonal formations could be applied successfully. However, the progressive imposition of an arid climate during the Early Holocene in the form of prolonged droughts motivated events of abandonment, relocation and incorporation of previously inhabited areas. In the long term, this led to the abandonment of most of the pre-puna sites and the occupation of the high puna and the desert floor.

The end of the Early Holocene was a period of deep transformations in social organisation. Technological changes reflect not only experimentation and learning on local resources but also the production of technologies to connect people in time and space, allowing the transmission of information. The complementary land-use of the highlands and desert lowlands was the result of a long process of landscape learning, which allowed to counteracted the greater spatio-temporal resource incongruence. In the long term, such transformations laid the foundations for an economic rationale which would form the basis of early social complexity in the region, and which persists down to the present.

11. Acknowledgments

We thank Francisca Santana and Mauricio Uribe for inviting us to present this work in the 2017 session of SAA, and to publish it in this volume. We also thank the anonymous reviewers for all their comments and suggestions. We thank Wilfredo Faúndez and Rodrigo Lorca for photographic support and Frank Haddon for his invaluable comments.

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Figures titles

Figure 1. Landscape variability of the south-central Atacama highlands: (A) Tuyajto lake (4000 masl); (B) Tulán river (3000 masl); (C) Tambillo desert lowlands (2200 masl).

Figure 2. Geographic location of south-central Atacama

Figure 3. Archaeological sites and environmental setting of the south-central Atacama: (A) Geomorphological morphoestructural units; (B) Zonal vegetation belts (after Villagrán et al., 1998); (C) Geomorphological profile (22ºS).

Figure 4. Paleoenvironmental and Paleoclimatic local records: (A) Estimated lake levels of Miscanti Lake (22º45 S, 4140 masl) (asterisk indicates a dry period) (Grosjean et al., 2001); (B) Groundwater discharge levels of Tilomonte wetlands (23.5ºS, 2550-2600 masl) (Rech et al., 2002); (C) Water table levels of Imilac-Punta Negra (Quade et al., 2008) (24-24.5ºS, 2900-3000 masl); (D) Rodent midden records of the Atacama basin (22-23ºS, 3100-3300 / 2400-3200 masl) (Latorre et al., 2003).

Figure 5. Archaeological sites: (A) TUI-5; (B) SPN-19/20; (C) TU-109; (D) TUY-1; (E) TA-1; (F) TUI-1.

Figure 6. Human events and climatic conditions: (A) ^{14}C dates (cal. BP) by elevation (masl); (B) Summed probability distributions. Dates have been calibrated with OxCal 4.3.2 (Bronk Ramsey, 2017) using the ShCal13 curve (Hogg et al., 2013).

Figure 7. Inferred pathway networks and placement patterns of archaeological sites

Figure 8. Site areas and available resources in a radius of 20 km.

Figure 9. Lithic raw materials distribution by site

Figure 10. Taxonomic diversity represented in TUI-5 (E-IV) (from Cartajena, 2003).

Figure 11. Retouched tools and other lithic artefacts: (A) Frontal scraper with lateral side-scraper and used edge (SPN-6); (B) Discoidal banded agathe (SPN-6) and unmodified quartz crystal (TU-68); (C) Frontal end-scraper (TUI-5); (D) ovoidal end-scraper; (E) micro-drills (TA-1 and TU-67); (F) Frontal end-scraper (SPN-19-20); (G) Lateral side-scraper (TA-1); (H) Frontal end-scraper (TA-1); (I) flat-flake with ultramarginal retouching (TU-68) (TA-1); (J) Rabot / facial core (TUI-5); (K) Mortar and handstone/pestle (TA-1).

Figure 12. Bifacial tools: (A) Non-stemmed, triangular “Tuina”; (B) Stemmed, barbed “Punta Negra”; (C) Stemmed with shoulders “Fishtail”; (D) Stemmed “San Martín”; (E) Biface; (F) Non-stemmed, triangular “Tambillo”; (G) Pentagonal.

Tables titles

Table 1. Archaeological sites and ^{14}C dates
Table 2. Locational patterns and environmental settings
Table 3. Projectile point type by raw material

Tables

Table 1

Site	^{14}C yr BP	cal yr BP (95.4% probability)	Lab. code	Dated material	Reference
TUI-1	10820±630	14178-10753	SI-3112	Charcoal	Núñez et al., 2005
	9080±130	10520-9709	NR	Charcoal	
TUI-5	10060±70	11805-11254	Beta-107120	Charcoal	
	9840±110	11614-10776	Beta 107121	Charcoal	
Tam-2/4a	9590±110	11190-10580			
TA-1	8590±130	10119-9144	Beta-25536	Charcoal	
	8870±70	10178-9631	Beta-63365	Charcoal	
TU-67	8190±120	9435-8663	Beta-25535	Charcoal	
TU-68	9290±100	10682-10231	Beta-25532	Charcoal	
TU-109	10590±150	12725-11995	Beta-142172	Charcoal	
	8870±50	10158-9692	Beta-188223	Charcoal	
SL-1	10280±120	12645-11716	HV-299	Charcoal	
	10400±13	12424-11405	N-3423	Charcoal	
	9960±125	11947-11107	N-3423	Charcoal	
TUY-1	8130±110	9395-8633	BeTA-105691	Charcoal	
	8210±110	9444-8771	Beta-105692	Charcoal	
AC-1	8720±100	10134-9487	Beta-105696	Charcoal	
SM-4	8130±50	9071-8650	Beta-116573	Charcoal	
SPN-1	9450±50	10775-10443	Poz-3274	Peaty sediments	Grosjean et al., 2005
	10460±50	12543-12035	B-8150	Peaty sediments	
	9180±50	10485-10206	B-8151	Peaty sediments	
	10350±60	12409-11830	B-8152	Peaty sediments	
	10440±50	12430-12020	B-8153	Vegetal	
	9230±50	10497-10240	B-8154	Peaty sediments	
	10470±50	12545-12050	B-8155	Peaty sediments	
SPN-6	10260±60	12068-11652	Beta-191578	Charcoal	
	10000±50	11693-11240	Beta-309834	charred material	Cartajena et al., 2014

SI-7	9940±50	11601-11204	Beta-309833	organic sediment	
	9950±50	11604-11220	Beta-309831	charred material	
SPN-19/20	9460±50	11065-10513	Beta-309833	charred material	
	9480±50	11070-10560	Beta-309828	Organic sediment	

Table 2

Site	Elevation (masl)	Vegetation belt	Settlement type	Geoform	Macro-geoform	Litho-stratigraphic unit
TUI-1	3117	Prepuna	Rockshelter	Hillside-ravine	Domeyko precordillera	CP-2. Continental marine and volcanic sequences (Carboniferous-Permian)
TUI-5	3174	Prepuna	Cave	Ravine bottom	Domeyko precordillera	CP-2. Marine and continental volcanic sequences (Carboniferous-Permian) Qa. Alluvial deposits, subordinately colluvial
Tam-1	2348	Desert	Open site	Phreatic beach	Atacama basin depression	Qa. Alluvial deposits, subordinately colluvial or lacustrine (Pleistocene-Holocene)
TU-67	2567	Desert	Cave	Ravine wall	Western cordillera	Qa. Alluvial deposits, subordinately colluvial or lacustrine (Pleistocene-Holocene)
TU-68	2454	Desert	Rockshelter	Ravine wall	Western cordillera	Qa. Alluvial deposits, subordinately colluvial or lacustrine (Pleistocene-Holocene)
TU-109	2962	Prepuna	Rockshelter	Ravine wall	Western cordillera	P3t. Ignimbrites (Pliocene)
SL-1	3264	Prepuna	Cave	Ravine wall	Western cordillera	PPl1c. Sedimentary deposits (Pliocene-Holocene)
TUY-1	4070	High-Puna	Open site	Lacustrine beach	Salars depression	P3i. Volcanic centers (Pliocene)
AC-1	4205	High-Puna	Open site	Lacustrine terrace	Salars depression	P3t. Ignimbrites (Pliocene)
SM-4	3695	High-Puna	Open site	Lacustrine terrace	Western cordillera	PPl1c. Sedimentary deposits (Pliocene-Holocene)
SPN-1	2978	Prepuna	Open site	Fluvial terrace and phreatic beach	Preandean basins	Qe. Aeolian deposits (Pleistocene-Holocene)
SPN-6	3018	Prepuna	Open site	Phreatic beach	Preandean basins	PPl1c. Sedimentary deposits (Pliocene-Holocene)
SI-7	3019	Prepuna	Open site	Phreatic beach and fluvial terrace	Preandean basins	Qa-alluvial deposits, subordinately colluvial or lacustrine (Pleistocene-Holocene)
SPN-19/20	2960	Prepuna	Open site	Playa freática	Preandean basins	Qa-alluvial deposits, subordinately colluvial or lacustrine (Pleistocene-Holocene)

Table 3

	Siliceous		Basalt		Obsidian		Tuff		Total	
Projectile Point type	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Fishtail		0,00%		0,00%	1	2,56%		0,00%	1	0,96%
Pentagonal		0,00%	2	6,06%	1	2,56%	1	14,29%	4	3,85%
Stemmed barbed	6	24,00%		0,00%		0,00%		0,00%	6	5,77%
Tetragonal (San Martín)		0,00%	20	60,61%	1	2,56%	1	14,29%	22	21,15%

Triangular, non-stemmed (Tambillo)	5	20,00%	3	9,09%	7	17,95%	3	42,86%	18	17,31%
Triangular, non-stemmed (Tuina)	14	56,00%	8	24,24%	29	74,36%	2	28,57%	53	50,96%
Total	25	100,00%	33	100,00%	39	100,00%	7	100,00%	104	100,00%