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Mestrado em Biologia Marinha

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Giulia Bocelli

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I Gabbiani

“Non so dove i gabbiani abbiano il nido,
ove trovino pace.
Io son come loro
in perpetuo volo.
La vita la sfioro
com'essi l'acqua ad acciuffare il cibo.
E come forse anch'essi amo la quiete,
la gran quiete marina,
ma il mio destino è vivere
balenando in burrasca.”

Vincenzo Cardarelli
(1887-1959)

Gaivotas

“Não sei onde as gaivotas fazem ninho,
onde encontram a paz.
Sou como elas,
em perpétuo voo.
Raso a vida
como elas rasam a água
em busca de alimento.
E amo, talvez como elas, o sossego,
o grande sossego marinho,
mas o meu destino é viver
faiscando na tempestade.”

Ttradução própria

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Abstract

The presence of humans and their activities are intensifying in coastal ecosystems leading to disturbance of wildlife and strong habitat modifications. Colonial seabirds that breed in coastal areas are an example of a particularly susceptible group to such pressures. Among the methods to assess the anthropogenic impact, physiological records can account for stress responses behind possible behavioral adaptations. We investigated if and how the anthropogenic presence in the reproductive colonies of the two sympatric species of seagulls, impact on their physiology and behavioral response. We selected the Yellow-legged gull (*Larus michahellis*) and the Audouin's gull (*Ichthyaetus audouinii*), due to their differential habits of interaction with humans. We recorded their heart rate during a designed walking-operator's disturbance experiment for a total of 6 days during incubation. With this aim we deployed artificial egg devices equipped with a microphone and a thermometer in 24 nests of each species, in two selected areas of each reproducing colony. We characterized each experimental nest by deploying a thermohygrometer to track the microhabitat temperature, recording vegetation maximum high and percentage cover, and using the dummy egg thermometer for nest chamber temperatures evaluation. Both species suffered an increase in heart rate just before leaving the nest unattended when disturbed by the operator. Contrary to expectation, the variation in heart rate response from undisturbed to disturbed conditions was similar between species. External factors such as the environmental temperature and the vegetation coverage proved to be related for both species, with the heart rate response, time necessary to resume incubation, and the hatching success. This study represents a first insight into the stress physiology of Yellow-legged and the Audouin's gulls and confirms the utility of the artificial egg as a minimal-invasive biologging tool for physiological research in incubating birds. These results lead us to hypothesize that global warming, extension of the human niche, and landscape modification, may threaten the reproductive success and fitness of colonial seabirds.

Keywords: yellow-legged gull, Audouin's gull, breeding seabirds, anthropogenic impact, artificial egg, heart rate, physiologic and behavioral responses.

Resumo

O aumento da população humana está a causar uma intensificação da perturbação na vida selvagem. A pressão antropogénica impõe uma pressão crescente na disponibilidade de recursos e espaço afetando, em consequência, a qualidade do habitat. Desta forma as espécies que possuem grande plasticidade e capacidade de adaptação estão provavelmente em vantagem neste ambiente em mudança. As zonas costeiras são um dos ecossistemas mais afetados por estas perturbações, devido à intensificação das atividades comerciais e turístico-recreativas. A forte presença humana ao longo das zonas costeiras promove uma redução dos espaços disponíveis para outras espécies, tanto pela expansão urbanística, como pela presença intensa de humanos em áreas outrora remotas. Áreas remotas, como falésias, praias e ilhas tornam-se assim áreas de sobreposição entre atividades de lazer e zonas de reprodução da vida selvagem. Desta forma, com esta investigação pretendemos avaliar o impacto da presença humana na reprodução de aves nestes locais remotos.

Entre as espécies que se reproduzem em zonas costeiras e ilhas remotas, as aves marinhas coloniais são as mais comuns e também as mais afetadas pela pressão antropogénica. As colónias reprodutivas de aves marinhas consistem geralmente em grupos de aves que constroem ninhos no solo. A presença humana nas colónias conduz frequentemente à destruição dos ninhos, ao espezinhamento de ovos e aves juvenis e causa também reações de stress agudo às aves adultas nidificantes. Por conseguinte, a presença antropogénica afeta frequentemente o tamanho e distribuição das populações de aves e altera o equilíbrio entre as espécies, favorecendo por vezes umas espécies em relação a outras.

A gaivota de patas amarelas (*Larus michahellis*) e a gaivota de Audouin (*Ichthyaetus audouinii*) são duas espécies que, embora ocupem nichos ecológicos semelhantes e por vezes se encontrem em situações simpáticas, diferem profundamente na sua tolerância aos humanos. A gaivota-de-patas-amarelas mostra um comportamento alimentar plástico e oportunista. Esta espécie explora recursos alimentares antropogénicos em cidades e em lixeiras e apresenta uma elevada capacidade de adaptação à presença humana e à paisagem modificada,. A gaivota-de-Audouin, por outro lado, tem um comportamento alimentar especializado e as suas interações com os humanos são quase exclusivamente relatadas para necessidades de alimentação, em particular, durante operações de descarte de pescado.

A maior parte da informação disponível sobre plasticidade e capacidade de adaptação destas duas espécies baseia-se na análise do comportamento alimentar em relação a atividades antropogénicas. No entanto, pouco de sabe sobre a forma como a presença humana afeta estas espécies de gaivotas durante as atividades de reprodução. A presença humana nas colónias nidificantes é normalmente detetada pelas aves adultas como a presença de um predador. Sabe-se que isto provoca uma reação

comportamental que vai desde deixar o ninho temporariamente sem vigilância ou mesmo à potencial deserção definitiva do ninho, até ao abandono permanente do local de nidificação para novas colónias reprodutivas. Deixar o ninho sem vigilância, mesmo por períodos curtos, pode levar a um risco acrescido de predação dos ovos e juvenis, e à sua exposição a stress térmico. Em geral, os estudos sobre o impacto das perturbações antropogénicas nas áreas de nidificação, concentram-se frequentemente nas respostas comportamentais.

As respostas comportamentais observáveis podem muitas vezes não ser suficientemente sensíveis para caracterizar os impactos negativos mais subtis, mas, que ainda assim, podem implicar custos fisiológicos e gastos energéticos. A avaliação das respostas fisiológicas, tais como níveis hormonais induzidos pelo stress e/ou aumento da frequência cardíaca, permitem uma avaliação mais sensível da magnitude da perturbação a nível individual. No entanto, os procedimentos necessários para adquirir dados fisiológicos podem interferir com a fiabilidade dos resultados. Técnicas como a colheita de sangue, aplicação de implantes cirúrgicos e aplicação de dispositivos externos, como os gravadores de eletrocardiograma, requerem frequentemente uma manipulação elevada da ave, o que pode levar a uma alteração da resposta do animal à perturbação humana. Este estudo teve por objetivo avaliar a presença do stress fisiológico como resposta à perturbação humana, em termos de frequência cardíaca, durante a incubação na gaivota-de-patas-amarelas e na gaivota-de-Audouin, usando ovos artificiais equipados com microfones como instrumentos de amostragem. Também queríamos avaliar a potencial diferença desta resposta entre as duas espécies de gaivotas. Formulámos a hipótese de diferenças devido à sua abordagem comportamental aos seres humanos. Neste estudo avaliámos também os potenciais efeitos negativos de tal perturbação humana sobre fatores que podem comprometer a viabilidade das ninhadas, tais como a temperatura dos ovos e a cobertura vegetal.

Selecionamos a ilha Deserta (ilha da Barreta), a sul de Portugal, como local de estudo porque aqui a gaivota-de-patas-amarelas e a gaivota-de-Audouin reproduzem-se em simpatria. Isto proporciona um sistema modelo único para comparação da sensibilidade destas duas espécies de gaivota a perturbações humanas durante a época reprodutiva, e também para compreender se existe uma base comportamental para a adaptação à presença humana, que possa ajudar a explicar as tendências das suas populações.

Conduzimos o nosso estudo na época reprodutiva de 2021 (de abril a julho) e estabelecemos duas áreas de amostragem para cada espécie – uma área controlo e uma área experimental. Após a identificação das quatro áreas, marcámos 24 ninhos nas áreas experimentais, e 38 nas áreas de controlo. Os 124 ninhos foram então caracterizados relativamente à sua cobertura vegetal, utilizando um quadrado de 1 m2. O nosso objetivo era avaliar a variação do ritmo cardíaco das aves em incubação e o tempo necessário para que as mesmas aves retomassem à incubação após a perturbação, como indicadores do stress comportamental e fisiológico. Avaliámos também a variação da temperatura dos ovos durante a perturbação e os consequentes períodos de ausência do ninho.

Realizámos a amostragem, com recurso ao ovo artificial, durante a segunda e terceira semanas de incubação dos 48 ninhos experimentais. O ovo artificial é um dispositivo eletrónico composto por um Arduino -, uma plataforma de hardware constituída por uma série de placas eletrónicas equipadas com um microcontrolador, um microfone, um sensor de temperatura e bateria. Para simular experimentalmente a perturbação antropogénica, caminhamos pelas áreas onde instalámos os ovos artificiais. A perturbação do operador foi concebida para seguir sempre a mesma rota num transecto definido - uma linha recta imaginária a não mais de 5 metros dos ninhos amostrados - duas vezes por dia, durante 10 minutos de cada vez, a um ritmo regular. Este procedimento foi repetido durante 6 dias seguidos para cada espécie, onde cada ninho foi amostrado duas vezes, com um intervalo de 3 dias. Durante cada dia de amostragem, foram adquiridos registos do ritmo cardíaco dos adultos em incubação e da temperatura da ninhada em 8 ninhos diferentes. Estes dados foram analisados através de dois softwares, o editor de áudio, denominado Audacity (versão 3.0.0) e um novo software (Laboratório de Instrumentação e Física Experimental de Partículas (LIP), Universidade de Coimbra, Portugal) especificamente desenvolvido para a utilização de ovos artificiais em pesquisas fisiológicas. Além disso, caracterizámos cada ninho experimental através da utilização de um termo-higrómetro para acompanhar a temperatura do micro-habitat, registo da máxima cobertura vegetal em altura e percentual, e através de uma câmara para gravar vídeo e integrar com as observações comportamentais presenciais.

A perturbação experimental levou os adultos nidificantes a fugir temporariamente do ninho como consequência de um aumento súbito do ritmo cardíaco. A diferença do batimento cardíaco da ave entre períodos em que não foi perturbada e durante a perturbação mostrou ser significativamente diferente tanto na gaivota-de-patas-amarelas como na gaivota-de-Audouin. Isto confirma que a presença antropogénica nas colónias reprodutoras é uma fonte de perturbação.

A nossa expectativa de encontrar uma diferença significativa na magnitude de resposta do ritmo cardíaco à perturbação não se verificou. O aumento relativo do ritmo cardíaco foi similar na gaivota-de-patas-amarelas e na gaivota-de-Audouin. Este resultado sugere que as diferenças de adaptabilidade e plasticidade entre estas duas espécies de gaivota não estão relacionadas com a sua sensibilidade em termos de resposta fisiológica de aumento do ritmo cardíaco face a um evento de perturbação humana durante a incubação em ambiente natural, pelo menos numa exposição de curta duração. Finalmente, os fatores externos, tais como as temperaturas ambientais e a cobertura vegetal, provaram estar relacionados em ambas as espécies, respetivamente, com a magnitude da resposta fisiológica em termos de batimento cardíaco e o sucesso da eclosão, além de imporem mudanças comportamentais

nas aves. Estes resultados levam-nos a colocar a hipótese que as alterações climáticas e consequente aumento de temperatura, a extensão do nicho humano e a modificação da paisagem, ameaçariam o sucesso reprodutivo e a sobrevivência destas espécies.

Esta investigação fornece uma primeira visão sobre a fisiologia do stress da gaivota-de-patas-amarelas e da gaivota-de-Audouin e confirma a validade do ovo artificial como uma ferramenta pouco invasiva para a investigação fisiológica das aves em incubação. São necessárias mais investigações se quisermos fornecer conhecimentos válidos para a gestão e conservação destas espécies, tal como de outras aves marinhas coloniais e o ambiente a que elas pertencem.

Palavras-chave: gaivota-de-patas-amarelas, gaivota-de-Audouin, aves marinhas reprodutoras, impacto antropogénico, ovo artificial, ritmo cardíaco, respostas fisiológicas e comportamentais.

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List of acronyms and experiment parameters

Transect 1/2 Virtual line that crossed each colony, to which the distance from each of the experimental nests did not exceed 5 m. It corresponds to the line walked by the researcher in order to cause the anthropogenic disturbance, for a total duration of 10 minutes. Time Transect 1: 10:00/10:30 h; Time Transect 2: 14:00/14:30 h

Sampling day Periods:
Period I It began with the deployment of the dummy egg (9:00-9:30 h) and finished 30 minutes after the end of the Transect 1 (around 10:00 -10.30 h)
Period II It began at the end of Period I and ended 30 minutes after the end of the Transect 2 (around 14:00 - 14.30 h)
Period III It started at the end of Period II and ended with the retrieval of the dummy eggs.

HR Heart Rate
DE Dummy Egg
TH Thermohygrometer

Stress HR
stress heart rate; obtained in the period of the transect between when the nesting gull, detecting the incoming operator's presence and perceiving it as a threat, showed the physiological preparation for a “fight or flight” reaction (detected by a rise in heart rate) until the gull took off (absence of heart rate signal).

Basal HR
The heart rate recorded in the absence of anthropogenic stimuli

SHRR
Standardized Heart Rate Response; calculated as ((Stress HR - Basal HR/ Basal HR) *100), and divided by the mean body mass of each species (*L. michahellis*: 1000g, Matos et al., 2018; *I. audouinii*: 600g, Verissimo, 2018).

Resume time
The time interval between the end of the transect and the resuming of the incubation

DE temperature variation_gull’s absence
Difference between the clutch minimum and maximum temperature recorded by the dummy egg during the Transect until the gull resumed incubation

DE temperature variation_10 min transect
Difference between the clutch minimum and maximum temperature recorded by the dummy egg during the 10 minutes of the Transect

TH temperature before transect/ disturbance
Mean nest microhabitat temperature during 5 minutes interval, before each transect, to assess the microhabitat temperature right before the gull took off

TH temperature transect
Mean nest microhabitat temperature during the 10 minutes of Transect

TH Temperature absence
Mean nest microhabitat temperature for the entire time of gull absence until it resumed incubation for each Transect

Source: *Giulia Bocelli*

General introduction

In 1800 the world population has been estimated to consist of 1 billion people. Since then, the human population has grown following an exponential curve, with a prevision of 8 billion people living simultaneously on Earth in 2023 (Roser et al., 2013). The speed and measure of this growth is requiring a constant increase in quantity of natural resources and physical space needed to sustain the human population. As result, natural resources are drastically decreasing for the industries intensification, as the availability of space, due to demographic and geographic anthropogenic extension (Boivin et al., 2016). These two conditions are, at the same time, reducing the availability of resources for the rest of living creatures and releasing discards and pollutants that are modifying the balances and cycles that underpin the Earth's ecosystems (Harte, 2007). Although this is a worldwide phenomenon , coastal ecosystems are under an even greater pressure because most of the human population lives in coastal areas (Lotze et al., 2006). Coastal areas represent 15% of the drylands on Earth, according to EEA (European Environment Agency) and, just in Europe, in 2016, already 85% of the population was living in the coastal zone. The prevision for 2025, is for 75% of the world population living within a zone of 100 km from the coast (EEA, 2020). This concentrated overpopulation results in a dramatic reduction of the ecological niches, consistently threating the wildlife of coastal ecosystems and increasing the niche segregation and depletion of animals like seabirds (Airoldi & Beck, 2007).

Seabirds are characterized and usually defined mainly for their strict lifetime relationship with the marine environment, where feeding is the major activity. Even though some species spend several months at sea, all seabirds rely on land for specific periods of their life, such as during early development and reproduction. During the breeding season, hundreds, even thousands of seabirds gather on beaches, cliffs and islands and establish reproductive colony sites (Schreiber & Burger, 2001). The association in breeding colonies in seabirds is a common behaviour (96% of, so defined, seabird species breed in colonies; Schreiber & Burger, 2001), to the point that scientists have elaborated several theories on the reason that makes this strategy advantageous. Among the most corroborated hypothesis, across species, is the protection from predators, facilitation on finding food and a better adjustment of breeding time to environmental conditions (Hamilton, 1971; Ward and Zahavi, 1973; Coulson, 1985; Furness and Monaghan, 1987). Despite these and other plausible advantages responsible for its evolution as a winning strategy, several examples in literature report colonial breeding as being highly costly in terms of energy (Moulaï et al., 2008; Ramírez et al., 2010; Van de Voorde et al., 2015). On top of the costs of reproduction, individuals in colonies suffer the disadvantage of an increased risk of parasites and disease, and a potentially high and constant, intraspecific and interspecific (in sympatric breeding situations) competition that can lead to predation and aggression (Burger, 1979; Schreiber & Burger, 2001). Competition in breeding colonies requires a time-energy share devoted to constant communication, reduced resting time, a potential increase in flying time for feeding necessities and even occasional displacement and predation fight (Burger, 1979; Schreiber & Burger, 2001).

Overall, to reproduce successfully seabirds must be in a good health condition. Additional demands on energy expenditure, such as responding to anthropogenic disturbance, may reach a certain threshold leading individuals to prioritize survival over reproduction (Ellenberg et al., 2013).

1.1 Seabirds breeding colonies and human disturbance

In this scenario it appears clear the importance of investigate and assess human disturbance during reproductive season. Several researches account for species that suffer population decline due to human disturbance effects during breeding season. Giese in a study of 1996, assessed a decline in the breeding productivity of Adeline Penguins (*Pygoscelis adeliae*) caused by repeated turistic human approaches. Aircraft and ecotourism are reported in few papers to have effects on pelican clutch size, hatching success and fledging success, lowering the reproductive success (Anderson and Keith, 1980; Boellstorff et al., 1988; Bunnell et al., 1981). Moreover, studies like the one conducted by Yasue and Dearden, on a population of Malasian polver (*Chaeadrius peronii*) in 2006, shows the effects that human modified-landscape can apport to vegetation structure which negative affects the reproductive success by rising predation risk. Finally, studies on Audouin's gull which had suffer population decline in past years revealed that the management and protection of this specie reproductive colonies from human disturbance, have led to global repopulation (Sarzo et al., 2008; Oro and Ruxton 2001; Oro et al., 1999).

According to Bolduc & Guillemette (2003) and Nisbet (2000), disturbance is definable as any human activity that induces, directly or indirectly, a physiological or behavioural change of one or more animals. Human-related disturbance in seabird breeding colonies can be classified in two categories: the habitat and ecosystem modifications derived from human activities and the physical presence of humans in the area (Boivin et al., 2016; Carney & Sydeman, 1999; Crowe et al., 2000).

The first disturbance type acts "at distance", changing the habitat conditions on a large-scale, that can bring a disadvantage in terms of time-energetic costs to the seabirds and reducing the quality and quantity of resources (Grémillet & Boulinier, 2009; Schreiber & Burger, 2001). To this regard, Pedro et al. (2013) in a study on the yellow-legged gull (*Larus michahellis*) diet across a century detected a significant decrease in its trophic position. Along a period of 89 years, the yellow-legged gull food resources changed markedly due to a reduction of natural food resources such as fish, apparently caused by human exploitation. This leads the yellow-legged gull to shift its diet to easier and more available prey such as anthropogenic waste.

The second disturbance type, human presence, creates an event perceived as equivalent to a predation risk, decreasing the individual time-energy budget available for other activities such as foraging, resting or incubating. In particular in this last circumstance human presence potentially decreases reproductive success, exposing the nest to unattendance, that higher risk of predation, abandonment and heat stress (Frid & Dill, 2002; Gill et al., 1996).

The effects of both kind of disturbance on individuals' fitness is highly variable which rise the difficulty and the needs of investigation. The geographic location, the frequency of the human activities and their duration over time, the species' behavioural and physiological capacity of adaptation, are the main drivers of the disturbance stimuli response (Nisbet, 2000). With this regards Burger and Gochfeld in 1999 assessed the ability of Laysan Albatross (*Diomedae immutabilis*) to habituate to human presence under repetitive stimuli. The adults and the chicks that experienced a greater rate of human disturbance approach were responding aggressively at closer distance then the one which were less used. In the same way an experiment conduct on Adeline Penguins by Fraser and Patterson in 1997, reveal that penguins exposed to a little contact with humans suffer more dramatic decline in population then those exposed to ecotourism over the same period.

The importance of studying the impacts of such disturbance stimuli set in ultimately effect of reduce reproductive success and contribute to the decline of breeding populations (Carney & Sydeman, 1999; Grémillet & Boulinier, 2009).

1.2 Habitat and ecosystem modifications effects

The massive presence of human beings and activities in coastal ecosystems results frequently in deep modification of the environment itself creating what is called a human-modified landscape (Boivin et al., 2016). The effects of anthropogenic landscape modification on seabirds breeding colonies lead, to a reduction in the availability of physical space for breeding – competition increases – and to the degradation of the ecosystem, in particular the vegetation structure of the area. Regarding this last condition, the study of Yasué & Dearden (2006) on Malaysian plovers, represents a clear example of how human modified vegetation structure can affect seabirds breeding success. In their research, the endemic low vegetation characteristic of reproductive colonies, composed by *Acacia* and mangrove, has been substituted by taller trees offering the nests an insufficient shelter from ground predators.

This anthropogenic modification led to the reduced reproductive success of Malaysian plovers (Yasué & Dearden, 2006).

On top of the potential higher risk of predation for themselves, the chicks, and the eggs, vegetation changes or reduction expose incubating birds to higher risk of thermal stress (Oswald & Arnold, 2012). Moreover, the increasing proximity of the colonies to human settlements and activities leads to a more frequent presence of humans in breeding colonies (see below) and may also affect the birds due to additional disturbances related to pollution, either chemical and/or noise pollution (Culik et al., 1990). Lastly, seabirds are usually located at the top of the food chain which means that all the contaminants (e.g. pesticides, chlorinated hydrocarbons, metals, and petroleum products) reach high levels of bioaccumulation on these birds (Burger & Gochfeld, 1993, 2004; Monteiro, 1996). This is one of the reasons why seabirds are used as bioindicators of ecosystem quality (Burger & Gochfeld, 2004).

Thus, each of the circumstances listed above of habitat alteration and/or degradation, increase the energetic costs for nesting seabirds to breed successfully and threat their chance of survive in what are, nowadays, novel environments created by human activities.

1.3 Direct human disturbance

The increase of human presence in coastal areas also derives from the increased proximity to human settlements, but also the growing amount of people that visit remote places and islands for touristic purposes. Sandy beaches, coastal islands and cliffs frequently coincide with wild animals' refugees or breeding locations, such as for ground nesting seabirds (Burger & Gochfeld, 1993; Carney & Sydeman, 1999). The human presence in the areas of seabird breeding colonies can be identified as recreational tourism, nature observation tourism or ecotourism, and research purposes (Diamond, 1985). We will focus on touristic intrusions, considering that scientific studies although not completely harmless, are deeply aimed to induce the least disturbance possible (Frederick and Collopy, 1989; Carney & Sydeman, 1999; Nisbet, 2000). Indeed, good practices should be applied when performing conscious research and in order to obtain valid and unbiased results (Carey, 2009; Rodway et al., 1996).

We will focus on both visually observable and non-observable effects of touristic disturbance in seabird breeding areas. Both of them eventually compromise the reproductive success of the colony but, while the first provoke a visually identifiable effect, reflected on e.g., adult behaviour or nest environment, non- visually observable effects refer to, for instance, physiological changes.

1.3.1 Visually - observable effects

People involved in recreational touristic activities such as walking, boating, other sport activities or picnicking, are likely unaware of crossing or passing by a nesting area (Knight and Cole, 1995; Giese, 1996). Conscious or not, their direct effects are reflected both on adults and nests (Lord et al., 2001; Pfeiffer & Peter, 2004). Ecotourism, in contrast, involves nature observation, including that of breeding colonies, and precautions are usually taken in order to avoid trampling and damaging nests, chicks and eggs accidentally (Burger, 1981; Burger & Gochfeld, 1998). However, Burger & Gochfeld (1993) calculated that *Sula dactylagra, S. sula*, and *S. nebouxii* nesting within two meters from the ecotourist trail, fled from the nest up to 95% of the visiting events, leaving the nest unprotected. This highlights the fact that even managed eco-tourism may lead to disturbance events.

In both cases, recreational or eco-tourism, seabirds perceive the incoming person as a predator's incursion into the colony, to which the ultimate reaction is to fly away (Burger, 1981; Frid & Dill, 2002; Tarlow & Blumstein, 2007). Whether the intent of flying away is to reduce the risk of predation or an attempt to intimidate the intruder, this behaviour is part of an anti-predator strategy (Riechert & Hedrick, 1990; Gill et al., 1996). Such strategy consists in a trade-of between the benefits of abandoning its current breeding activity and reacting to the predator presence with inherent energetic costs, or keeping in its position and activity and risking death (Gill et al., 1996; Frid & Dill, 2002). The magnitude of this reaction is frequently analysed in researches thought flight-initiation distance (FID) and resume incubation time parameters. FID consists in evaluating the distance between the disturbance source and the nesting seabird at which the seabird flies away, (FID; Tarlow & Blumstein, 2007) and represent a direct indexes of the acceptable trade-off threshold of the bird, between energy saving and survival (Barter, 2004; Tarlow & Blumstein, 2007). Resume incubation time is the time the nesting seabird takes to resume incubation (Burger, 1981), when it feels safe. Measures of FID and resume incubation time provide a main technique to survey the behavioural response to human presence and therefore the effect magnitude.

Fleeing behaviour has an affect also on the viability of the nest contents. In absence of the adults, the amount of chicks survival and egg successfully hatched are directly threatened by the predation risk and detrimental effects of thermal stress (Amat & Masero, 2004; Oswald et al., 2008; Yasué & Dearden, 2006). Therefore, the probability of reproductive success became proportional to the time the adults' spend out of the nest (Anderson & Keith, 1980; Bolduc and Guillemette, 2003). Breeding success parameters such as hatching success, chicks' survival, clutch size, and number of fledglings are used to quantify the observable effects of human disturbance on reproduction (Tarlow & Blumstein, 2007) as FID and resume incubation time quantify the behavioural changes.

1.3.2 Indirectly observable effects

While the survey of breeding success is a valuable tool to monitor the impact of anthropogenic disturbance on populations, the evaluation of the stress impact on the individual, as bare behavioural observation, cannot be complete without a physiological analysis (Culik et al., 1990; Nimon et al., 1996). The energy-time trade-off can lead to a reduction of the behavioural anti-predator response, especially in circumstances where the energy demand and investment are already high (Dawkins & Carlisle, 1976; Nimon et al., 1995; Gill et al., 2001). Complementary physiological assessment become essential, as they can enable detection of subtle effects even before those are observable at the population level – which in some case may be already too late (Gill et al., 2001; Martin et al., 2015). With this regard, Culik et al. (1990) showed that the behavioural response to avoid human disturbance, in a colony of Adelie penguins (*Pygoscelis adeliae*), was reduced from the initial stages of incubation in comparison to the period closer to the hatching time. These results could have suggested an adaptation of seabirds to a repeated disturbance stimulus over time. However, cardiac rate data recorded within the same study, demonstrated a significant heart rate increase under human proximity stimuli, for the entire reproductive period (Culik et al., 1990). The absence or reduction of the observable behavioural response - particularly in established nests - is, therefore not reliable or sensitive enough to evaluate disturbance effects and habituation (Borneman et al., 2014; Ellenberg et al., 2009; Nimon et al., 1995). In another more general prospective, colonial birds that avoid anthropogenic disturbance by deserting entire reproductive sites are commonly considered - under a behavioural point of view - more threatened by human presence then those that persist on the breeding sites under perturbance. However, as point out by Gill et al., in 2001, those groups or species who stays could be force in it by the absence of alternative reproducing sites, and therefore exposed to high level of stress under human disturbance, more than those who actively avoid it. The behavioural response of an animal to a disturbance -which depends on the energy-time devoted to the breeding attempt up to the moment the stimulus occurs, the nature of the stimulus, habitat circumstances, individual characteristics, experience, and alternative reproductive sites - can lead easily, to misinterpret the anthropogenic impact, especially when not related to survival and reproductive success data (Ellenberg et al., 2009; Gill et al., 2001).

With this prospective, physiological responses avoid behaviour interpretation providing objective parameters to detect alterations under stimulus. Moreover, physiological responses influence on individual fitness is well documented, furnishing an even less subjective interpretation of the stimulus impact then behavioural responses (Ellenberg et al., 2006).

The impact of disturbing stimuli, in physiological analysis, is usually evaluated as the magnitude of parameter variation between undisturbed and disturbed conditions. This variation when present represent the physiological stress magnitude (Sapolsky, 1990). Physiological stress response requires the engagement of high levels of energy promoted by the sympathetic nervous system, to prepare the body for a "fight or flight" reaction (Culik et al., 1990; Fowler, 1999). This reaction is natural and generally adaptive and prepares the individual to face danger and survive (Müller et al., 2017). However, if the stimulus persists and habituation does not occur, the organisms enter a state of chronic stress where the alteration of physiological parameters persist. This leads to the inhibition of vital functions in consequence of the energy devoted to cope with stressors. Physiological responses therefore, allows to records stress responses even when is not reflected in the behaviour and to quantify and monitoring it through time and under different stimuli, by establishing basal physiological referential values in absence of stimuli (Culik et al., 1990; Tarlow & Blumstein, 2007 see *Glucocorticoid* and *Cardiac Response* paragraphs). Through this method, Fowler (1999) studied the adrenocortical response to stressors in Magellanic penguins (*Spheniscus magellanicus*) used to different level of human presence. Fowler observed that the variation in adrenocortical hormone levels was greater for the group of bird breeding in remote areas, and therefore less accustomed to see humans. The pinguins who nest in areas under high touristic presence on the other hand, did not report any alteration in hormonal levels when approached by the researchers. This study therefore, demonstrate the occurrence of habituation for one group of pinguins and not for the other under the same potential stressor, based on the magnitude of response in relation to basal level using a physiological metric.

Hormonal parameters (such as glucocorticoids) and heart rate telemetry are the two most common methods to acquire data on physiological responses to stressors (Tarlow & Blumstein, 2007). Weisenberger et al. in 1996, studied ungulated heart rate response to assess the impact of aircraft. In the same way Bisson et al. (2009), Culik et al (1989) and Wilson et al. (1991) using heart rate telemetry showed in stress reaction to human approach in White-eyed Vireos (*Vireo griseus*) and Adelie Penguins. Flower in 1999, use a hormonal approach to assess the impact of ecotourism in a colony of Magellanic penguins, revealing a capacity of habituation. Physiological parameters revealed to be useful tools also to discern impacting stimuli from those that are not. For instance, Harms et al. (1997) and Borneman et al. (2014) showed that aircraft flying over the colonies of Black Ducks (*Anas rubripes*) and American Oystercatchers (*Haematopus palliatus*) have no or minimal effects on the heart rate of the birds, besides what could be expected. The potential of recognize for specific species and colonies threatening stimuli from non-threatening once, allows precised and focused management plans, that could actually provide a conservation of the specie without interfering where not necessary with human activities.

1.3.2.1 Heart rate telemetry

The cardiac response has been used for over 30 years as a sensitive and precise tool to evaluate human disturbance and quantify its relative impact on wild animals (Culik et al., 1990; Ellenberg et al., 2006, 2013; Giese et al., 1999; MacArthur et al., 1979; Müller et al., 2017; Nimon et al., 1996). It allows comparing the magnitude of the impact of different stimuli and the potential effects on individuals, colonies and species in terms of survival and reproductive success (Borneman et al., 2014; Ellenberg et al., 2013). In this regard, Ellenberg et al. (2013) measured the heart rate of incubating Yellow-eyed penguin (*Megadyptes antipodes*), under different conditions including natural stimuli, as during calling behaviour and nest maintenance, and in response to human disturbance, from walking in the nesting area to individual handling. Cardiac measurements are, therefore, an important tool for defining as a quantity, individuals response to specific stressors and document the time it takes to recover from a particular stressor, in a sensitive way (Ellenberg et al., 2009, 2013; Giese, 1998). An evaluation of heart rate levels during resting periods (=basal heart rate), and during disturbance stimuli as well as the time taken to recover from such disturbance events can be used to detect habituation, stressful conditions and/or chronic stress (Culik et al., 1990; Nimon et al., 1996; Tarlow & Blumstein, 2007).

Lastly, the cardiac response after validation may be used as a proxy for metabolic rate, and to predict oxygen consumption and energy expenditure (Tarlow & Blumstein, 2007). This requires, however, higher levels of manipulation and the study of many individuals (Bevan et al., 1994; Weimerskirch et al., 2002).

1.3.2.2 Dummy egg

Physiological stress response provoked by anthropogenic disturbance stimuli, has already been proven in literature to cause lower fitness of individuals and therefore, reducing the reproductive success, by imposing a higher energy demand (Borneman et al., 2014; Nimon et al., 1996). External electrocardiograms (ECG) units and implanted electrocardiograms electrodes were used in human disturbance investigations, aimed to record the physiological stimuli impact on heart rate parameters (Giese, 1998; Stout & Schwab, 1980; Harms et al., 1997; Wilson et al., 1990). However, these survey instruments require animals' manipulation, from simple handling to surgical implantation. In animal experimentation it is necessary to reduce the manipulation and stress induced to the animals to a minimum, also because potential harm and preventive stress in the animal, related to human presence, may lead to bias in subsequent surveys (Borneman et al., 2014; Giese et al., 1999; Nimon et al., 1996). Because heart rate quantifies the physiological reaction to any external stimulus (Nimon et al., 1996) its monitoring should be performed in the least invasive way. Nimon et al. (1996) developed a heart rate sensor, egg-shaped, device: an artificial egg. The usage of a dummy egg deployed in the nest showed to allow for a minimum invasive survey: Adult manipulation is avoided, the egg camouflage does not affect adults' nest perception, and the only interaction during sampling, occurs when the egg is deployed and retrieved from the nest (Arnold et al., 2011; Giese et al., 1999; Nimon et al., 1996; Tarlow & Blumstein, 2007). Since then the dummy egg approach has been employed in several research projects on human disturbance evaluation during avian incubation (Arnold et al., 2011; Borneman et al., 2014; Ellenberg et al., 2006, 2013). This device provides instant records of heart rate through sound and in most cases allows the natural egg's rolling. Because the artificial egg is a contact-based device, it loses the signal in the moment that the bird's chest detaches from the microphone (Tarlow & Blumstein, 2007). Giese et al. (1999) validate ECG devices accuracy during a human disturbance experiment, by comparing the results obtained with the data recorded by a dummy egg. Finally, the dummy egg can be combined with the use of cameras and additional devices to relate habitat circumstances and birds' behaviour with the records, maintaining the minimal-invasively characteristic of the tool (Carney & Sydeman, 1999). The main disadvantage of this method is the fact that it can only be used during the incubation period.

1.4 Objective of this study

The present study aims to analyse in two model-species' of seagulls, the effect of direct human disturbance, combined with microhabitat characterization, during the reproductive season of 2021, to investigate their sensitivity to anthropogenic disturbance, in terms of physiological and behavioural responses. We evaluate the stress response to human disturbance in the yellow-lagged gull, *Larus michahellis* (Naumann, 1840) and Audouin's gull, *Ichthyaetus audouinii* (Payraudeau, 1826). The research will be based on two main questions: (1) do the yellow-legged gull and Audouin's gull have a physiological response to human presence in their nesting colonies during incubation?

Given that while the yellow-lagged gull opportunistically exploits novel human habitats and food resources such as cities and refuse dumps, easy readapting habits, Audouin's gull keeps its distance from humans and shows little evidence of adaptation to a human-modified landscape: (2) do the physiological response to human presence in the colonies during incubation, differs among the two model-species? In addition to these questions, we aim to evaluate (3) how the human disturbance can potentially affect the clutches' viability, exposing the nest to the consequences of been unattended and understand (4) if the nest vegetation characteristics has an influence on it. Taking in consideration the global change scenario and the human-landscape modification.

Overall, this study aims to evaluate the physiological and behavioural effects caused by human disturbance on two species of sympatric breeding colonial gulls, and assess the possible differences in the physiological response between the two species, based on the different human-related behaviour. We also aim to investigate the extent of potential detrimental consequences of such disturbance on hatching success.

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2 Manuscript

Anthropogenic disturbance effects on two sympatric species of gulls, *Larus michahellis* & *Ichthyaetus audouinii*, during the breeding season in Deserta island (Portugal)

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Keywords: Yellow-lagged gull, Audouin's gull, breeding seabirds, anthropogenic impact, artificial egg, heart rate, physiologic and behavioral responses.

2.1 Abstract

The presence of humans and their activities are intensifying in coastal ecosystems leading to disturbance of wildlife and strong habitat modifications. Colonial seabirds that breed in coastal areas are an example of a particularly susceptible group to such pressures. Among the methods to assess the anthropogenic impact, physiological records can account for stress responses behind possible behavioral adaptations. We investigated if and how the anthropogenic presence in the reproductive colonies of the two sympatric species of seagulls, impact on their physiology and behavioral response. We selected the Yellow-legged gull (*Larus michahellis*) and the Audouin's gull (*Ichthyaetus audouinii*), due to their differential habits of interaction with humans. We recorded their heart rate during a designed walking-operator's disturbance experiment for a total of 6 days during incubation. With this aim we deployed artificial egg devices equipped with a microphone and a thermometer in 24 nests of each species, in two selected areas of each reproducing colony. We characterized each experimental nest by deploying a thermohygrometer to track the microhabitat temperature, recording vegetation maximum high and percentage cover, and using the dummy egg thermometer for nest chamber temperatures evaluation. Both species suffered an increase in heart rate just before leaving the nest unattended when disturbed by the operator. Contrary to expectation, the variation in heart rate response from undisturbed to disturbed conditions was similar between species. External factors such as the environmental temperature and the vegetation coverage proved to be related for both species, with the heart rate response, time necessary to resume incubation, and the hatching success. This study represents a first insight into the stress physiology of Yellow-legged and the Audouin's gulls and confirms the utility of the artificial egg as a minimal-invasive biologging tool for physiological research in incubating birds. These results lead us to hypothesize that global warming, extension of the human niche, and landscape modification, may threaten the reproductive success and fitness of colonial seabirds.

Keywords: Yellow-lagged gull, Audouin's gull, breeding seabirds, anthropogenic impact, artificial egg, heart rate, physiologic and behavioral responses

2.2 Introduction

The consistent on-going of human population grow is exposing the ecosystems and wildlife of our planet to a rising disturbance pressure (Sodhi and Ehrlich, 2010). Changing on environmental temperature and vegetation distribution, landscape modification and reduction of pristine spaces are among the main consequences of the anthropogenic capillary extensions and derived activities (McDonnell & Hahs, 2015). Even though most of the human activities nowadays do not aim to harm wildlife, and are therefore considered non-lethal, wildlife animals still perceived humans as predators (Frid and Dill, 2002). The response of wildlife to this perception of anthropogenic presence varies drastically from one spice to another, as between populations. Indeed, while some species are threatened by drastic population decrease others fast adapt to take great advantage by the changing conditions (Tablado and Jenni, 2015; Carney and Sydman, 1999).

Across disturbance sources, in particular the increase of commercial and touristic-recreational activities collectively, is leading to an intensified presence of disturbance stimuli in those zones which are naturally nesting areas of seabirds' colonies (Yasué & Dearden, 2006). The capillary and growing presence of anthropogenic stimuli in these areas, imposes a growing pressure on reproduction site stability, physiology, reproductive behaviour and reproductive success of colonial seabirds (Carney and Sydman, 1999). This requires from species great plasticity and the ability to adapt, in times that transcend the evolutionary time scale (Parra-Torres et al., 2020). Therefore, anthropogenic presence frequently reshapes in size and distribution the populations and changes the balance between species, advantaging one over the others (Pfeiffer and Peter 2004; Carney and Sydeman, 1999; Nisbet, 2000; Martín et al., 2015).

Literature frequently reports seagull species to be betwixt the most plastic and opportunistic seabirds as scavengers and predators (Parra-Torres et al., 2020; Estévanez & Aparicio, 2019; Nisbet, 2000; Swennen, 1989). However the response to anthropogenic activities and presence changes substantially among species and population (Mendes et all., 2018; Calado et all., 2018; Van de Voorde et all., 2015; Carney and Sydeman, 1999; Burger, 1981; Ramírez et al., 2015): The yellow-legged gull (*Larus michahellis*; Naumann, 1840) and the Audouin's gull (*Ichthyaetus audouinii*; Payraudeau, 1826) are often studied in comparison when evaluating anthropogenic effects on their breeding populations, because they often nest in sympatry and deeply differ in their tolerance to humans (Calado et al., 2018; Matos et al., 2018; Moniz, 2015; Laranjeiro et al., 2020). The yellow-legged gull niche expansion and the changes in distribution observed in the Mediterranean populations in the last three decades (as for other large Laridae spp. in other continents, e.g., *Larus californicus* – Burns et al., 2018; *Larus argentatus* – Hatch, 1996) are thought to be mainly due to anthropogenic food resources availability (Calado et all., 2020; Duhem et al., 2008; Bosch et all., 1994; Oro et all., 1995). In contrast, the significant reduction around the 1960s-1970s of the Audouin's gull Mediterranean populations is attributed to negative response of the specie to the anthropogenic scenario (Perez et al., 2009; Bradley, 1988; Oro & Ruxton, 2001; Burger & Gochfeld, 1996). The yellow-legged gull shows plastic and an opportunistic feeding behaviour. This species exploits human resources, in cities and refuse dumps, and easily adapts to human-modified landscape and human proximity when this represents a favourable habitat in terms of energy (Mendes et al., 2018; Parra-Torres et al., 2020; Calado et al., 2018). Audouin's gull is a specialized feeder and its interactions with humans are almost exclusively reported for feeding needs, in particular, during fisheries discard operations (Oro & Ruxton, 2001; Navarro et al., 2010).

Most of the information available on plasticity and adaptation capacity of these two species is based on feeding behaviour analysis in relation to anthropogenic activities (Calado et al., 2018; Matos et al., 2018; Moniz, 2015; Laranjeiro et al., 2020). However, less is known about how human presence affects these gull species during breeding activities, especially when occur in their natural habitat.

Human presence in seabird nesting colonies is widely investigated and generally reported as a driver of population declines (Martín et all., 2015; Frid & Dill, 2002; Robert & Ralph, 1975; Carney & Sydman, 1999). In fact, intense human presence in nesting areas proved to impact the majority of seabirds species' reproductive success (Pfeiffer & Peter 2004; Burger & Gochfeld 1993; Carney & Sydeman, 1999). Human-induced negative impacts in nesting areas include vegetation changes, that affect protection from predators and thermal stress, egg trampling, and physiological and behavioural changes in nesting adults (Carney & Sydeman, 1999; Nisbet, 2000). The human presence in the nesting colonies is primarily perceived by the seabirds as a predator income (Frid & Dill, 2002). This is known to provoke the behavioural reaction that goes from temporarily leaving the nest unattended to potential definitive desertion of the nest, until permanent avoidance of the nesting site for further reproductive seasons (Carney et al., 1999; Nisbet, 2000; Burger, 1981; Medeiros et al., 2007). Leaving the nest unattended even for short periods may lead to an increased risk of predation of the eggs and chicks and their exposure to thermal stress (Borneman et al., 2014; Amat & Masero., 2004; Burger, 1981; Van de Voorde et al., 2015). For these reasons, the longer the adults' absence from the nests, the higher is the probability of reproduction failure (Carney, 1999; Burger, 1981). The magnitude of these anthropogenic impacts depends on type of disturbance, duration and frequency, resources availability, incubation stage but also individual features, such as sex, age and previous experience (Borgmann, 2011).

In general, studies on anthropogenic disturbance impacts in nesting areas, frequently focus on the behavioural responses. However, due to this array of potentially confounding factors, the lack of behavioural reaction does not necessarily mean a lack of impact (Nimon et al., 1996; Culik & Wilson, 1991). Visually observable behavioural responses might not be sensitive enough to characterise negative impacts, as habituation patterns. The assessment of physiological responses, such as stress-induced hormone levels and/or heart rate increase, may allow a higher resolution evaluation of the disturbance magnitude at the individual level (Tarlow & Blumstein, 2007; Borneman et all., 2014; Weimerskirch et al., 2002; Bevan et al., 1994). Physiological response can lead to a reduced fitness and investment in nesting duties from the adults, by increasing the oxygen consumption and energy expenditure (Bevan et al., 1994; Weimerskirch et al., 2002). Studies such as the one conducted in 2013 by Ellenberg et al., used heart rate methodology to assess the population rate of disturbance suffered under different stimuli. This study also asserted the sensitivity of the heart rate monitoring in evaluate how the impact of a specific disturbance, change and differs across time and repeated stimulus.

Nevertheless, the procedures required to acquire physiological data can interfere with the results reliability. Blood extraction, surgical and applicative implantation of external devices as the electrocardiogram's recorders, often require hight level manipulation that can lead to bias the further response of the animal to human disturbance or even bare presence (Culik et al., 1990; Borneman et al., 2014; Giese et al., 1999; Weimerskirch et al., 2002). Therefore, especially for nesting birds heart rate analysis, is growing the use of a minimal-invasive tool known as dummy egg, that minimize the bias produced by previous experiences, highly reducing the bird-human interaction needed to set up the tool for further recording (Giese et al., 1999; Arnold et al., 2011; Nimon et al., 1996).

In this study, we evaluated physiological stress response to human disturbance in terms of heart rate in incubating Yellow legged gull and Audouin's gull breeding in sympatry in Deserta island (Barreta island) in south Portugal. This provides a unique model system to experimentally compare the sensitivity of these to gull species to human disturbance and help to understand if there is a behavioural basis to human presence adaptation that may help to explain their population trends. We simulated human disturbance through walking transects in the middle of the breeding colonies and used heart rate loggers in the form of dummy eggs to assess changes in heart rate. We also used a tailored software to automatically extract the sound-based recordings as heart rate. We expect that during the experimental perturbation, the heart rate of both species will increase in relation to the heart rate recorded during non-disturbance periods (a). We expect the change in heart rate to be of lower magnitude in the yellow-legged gull in comparison with Audouin's gull, and that Audouin's gull will take a longer time to resume its incubation duties after disturbance (b).

We also assessed the potential negative effects of such human disturbance on factors that may compromise the clutches' viability. Therefore, we expect that the range of variation in the egg temperature during the adult’s absence after disturbance will be correlated with the time that adults take to resume incubation (c).

Overall, this study aims to evaluate the physiological and behavioural effects caused by human disturbance on two species of sympatric breeding colonial gulls, and assess the possible differences in the physiological response between them that may underline their adaptation capacity to a changing environment.

2.3 Material and methods

2.3.1 Study area

We conducted our study on Barreta Island (36°57’40, 7°53’20), one of the five barrier islands that enclose the Ria Formosa lagoon. This ecosystem, which extends for 184 km^2, is located on the south coast of Portugal, in the region of Algarve. Barreta Island, locally known as Ilha Deserta, is a 7 km length island, separated from the peninsula of Ancão (Ceia et al., 2010) and Culatra Island, by two artificial inlets on the West and East side, respectively (Figure 1). Inhabited until the last century, Barreta Island today hosts major recreational and touristic human activities during summer. The island access, located on the East side peer, converges the recreational activities in the area close to the restaurant (the only human construction still present) and on the closest beaches. Anthropogenic activities out of the summer season include almost exclusively research-related activities. Therefore, the anthropogenic footprint is confined to the eastern tip of the island.

The island is characterized by sandy dunes, with endemic medium to low vegetation, mostly bushes that cover and protect the dunes from the strong winds, and barely offer a repair from the sun (Herrero et al., 2020). The fauna of the Island includes cats introduced by humans, small terrestrial mammals, and birds, especially during the reproductive season (Filipe & Mendes, 2017). The reduced human presence compared with the surrounding areas of Ria Formosa and the other barrier islands, the presence of gull breeding colonies together with the low cover and low protection but endemic vegetation characteristics offer an excellent opportunity to evaluate the hypothesis of this research (Matos et al. 2018; Filipe & Mendes, 2017).

2.3.2 Study species

Yellow-legged gull (*Larus michahellis*)

The yellow-legged gull, *Larus michahellis* (Naumann, 1840), is one of the most widespread large seagulls (body mass 800-1 550g) all around the coast of Europe, with an increasing area of distribution that goes nowadays from the shores of the Black and Caspian seas until the north-eastern coast of the Atlantic Ocean (BirdLife International, 2021a). Its distribution along the year can vary due to its partially migratory habits, however, niche availability and abundance of resources linked to their opportunistic feeding behaviour are likely to lead to fairly sedentary habits (Kralj, et al., 2014; Serra et al., 2016). The interaction of yellow-legged gull with humans and anthropogenic activities has been well studied and consistently shows, in addition to the population increase and relative niche extension (Duhem. et al., 2008), a rising urban adaptation of this species justified by the opportunistic feeding behaviour and the habituation capacity (Oliveira, 2020; Lopes et al., 2021; Pais de Faria et al., 2021). Despite, some studies reporting the use of urban nesting grounds, their interactions with humans and their activities are mostly related to food resources, as breeding still mainly occurs on rocky cliffs and sandy islands (Duhem et al., 2008), as in the case of Deserta Island.

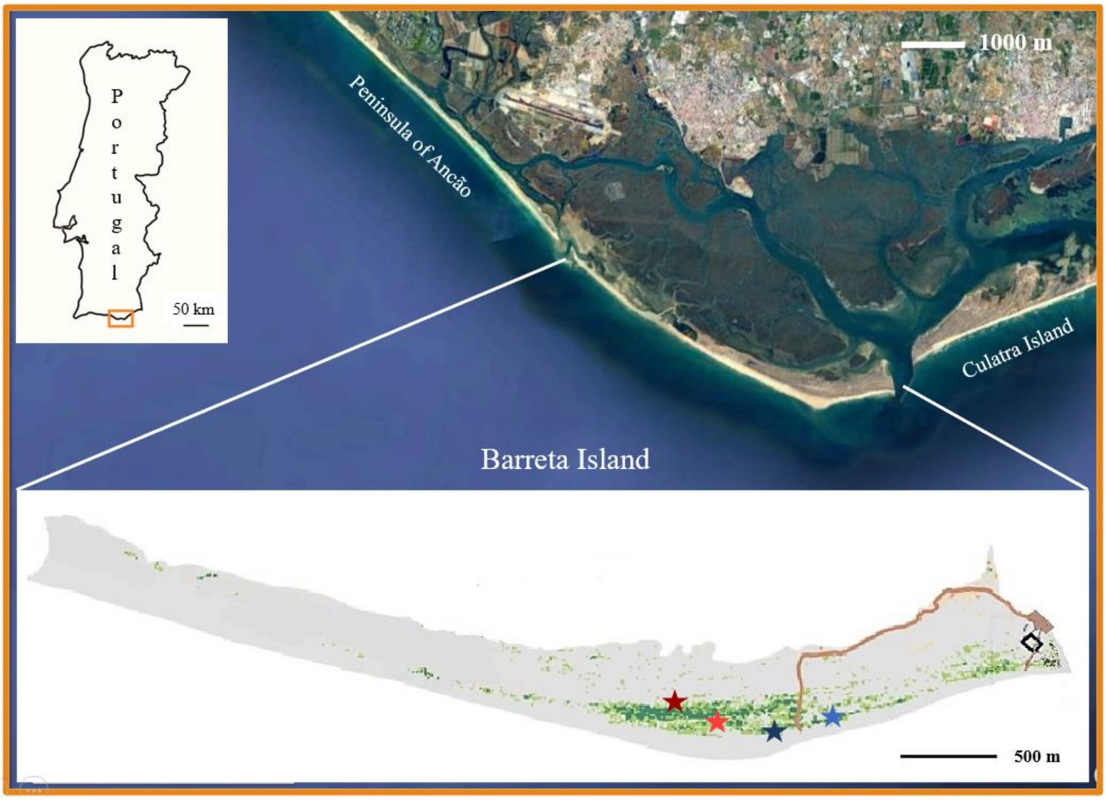

***Figure 1** Study site - Deserta (or Barreta) island, located in the south coast of Portugal (36°57'40, 7°53'20) - one of the five barrier island that divides the Ria Formosa Lagoon from the Atlantic Ocean. The restaurant is represented by the black square and the footpath is indicated in brown. The four stars represent the four study areas: yellow-legged gull (Larus michahellis) experimental zone (dark red star) and control zone (light red star) and Audouin's gull (Ichthyaetus audouinii) experimental zone (dark blue star) and control zone (light blue star).*

The breeding season and the colony size vary according to the surrounding environmental conditions. On the oceanic coast (e.g., Deserta Island) breeding occurs from March-April, when the pair starts to build the nest, until the end of July, when the juveniles fledge (at about 6 weeks of age; Matias & Catry, 2010; Serra et al., 2016). The nest consists of a sand shallow scrape reinforced by woven timber in sandy dunes and, on the cliffs, in rocks lined with plant material giving the classic cup shape (Kildaw, 1999). The nest's attendance and egg incubation, as well as the nest construction, is a duty shared by both parents that switch incubation shifts throughout the day in order to feed, cool down and rest. The clutch, usually consisting of 2 to 3 eggs (mean volume 69.4-84.0 mm^3 Vicente et al., 2012) laid in sequential days, hatch 28-30 days after the laying of the first egg (Serra et al., 2016). In case of failure of the first breeding attempt – due to competition, predation, and/or human disturbance, which are among the major causes - the monogamous pair may attempt a second nest within the same season (Yasué & Dearden, 2006).

Audouin's gull (*Ichthyaetus audouinii*)

The Audouin's gull, *Ichthyaetus audouinii* (Payraudeau, 1826), is a medium-size seagull (body mass 600-700g) endemic to the Mediterranean Sea (BirdLife, 2021b) and linked to yellow-legged gull by sympatric breeding circumstances (Calado et al., 2018). Even though they often share the same breeding habitat, the nest colony limits of each species remain well defined. The breeding colony of Audouin's gull follows a cluster pattern underling a reduced intraspecific competition and predation compared to yellow-legged gull whose nests are more dispersed within the breeding area, due to the high levels of competition (Estévanez & Aparicio, 2019).

Audouin's gull is capable of nocturnal vision; characteristic responsible for the different timings of feeding between the two species which creates a partial mismatch in feeding habits (Navarro et al., 2010). For instance, while yellow-legged gull usually feeds during the daytime, Audouin's gull takes more advantage of the night-time, allowing a reduced competition for foraging (Estévanez & Aparicio, 2019). Moreover, the nocturnal vision and the specialized feeding behaviour potentially decrease the interaction of Audouin's gull with human presence and activities. In fact, this interaction mostly occurs when this species feeds on fisheries' discards, a behaviour that is overtaking the specialistic foraging habits over the years (Navarro et al., 2010). It is reasonable to think that this reduced interaction and habituation to humans - together with the rising level of disturbance produced by resource exploitation and habitat degradation, due to anthropogenic activities – caused the populations' reduction observed in the 1970s and the subsequent classification of Audouin's gull as an endangered species (Burger & Gochfeld, 1996). However, nowadays, this process is being reversed, most likely because of conservation programs and the development of the species habituation, with a niche extension that overcomes the native Mediterranean Sea area (Oro & Ruxtuon, 2001). In fact, until the 2000s there were no records of Audouin's gull breeding sites in Portugal (oceanic coast) while today the colony of Barreta island - still the only one present in the country - is so well established that in 2021 registered a consistently higher number of breeding pairs (~4000) than that of yellow-legged gull (450 bp.) (J. A. Ramos; personal communication). Regarding the relationship of these two species in the breeding colony, their major interaction is due to predation and/or competition of yellow-legged gull towards Audouin's gull (Calado et al., 2018). The breeding sympatry condition of yellow-legged gull and Audouin's gull, and the contrast in their respective behavioural and feeding habits, provide useful comparative study models to assess the effects of anthropogenic disturbance.

The breeding season of Audouin's gull begins slightly later than that of yellow-legged gull (end of April - beginning of May) but also ends in July (Matias & Catry, 2010). The clutch, which is cared for by both parents, has an average of 3 eggs (mean volume 56.67-61.58 ml; Genovart et al., 2003) that hatch on average 28 days after the first egg is laid (BirdLife, 2021). Audouin's gull may attempt a second nest in the same season if the first attempt fails.

2.3.3 Nest Monitoring

We conducted our study during the breeding season of 2021 (April to July) in Barreta Island, where we established two sampling areas for each species – one control and one experimental (Figure 1). After the identification of the four areas, we marked 24 nests in the experimental areas, and 38 in the control areas, by placing a numbered wooden pole (Nest ID) close to each nest at the time the first egg was laid.

In the experimental areas we randomly selected the nests that were within 5 m of distance to a virtual line crossing the colony for the meters needed to achieve the 24 total nests for area (145 m for the Yellow-lagged gull and 45 m for Audouin's gull). Any nest further than 5 m from the virtual line has been excluded to assure equal and effective disturbance during the experiment. We continued

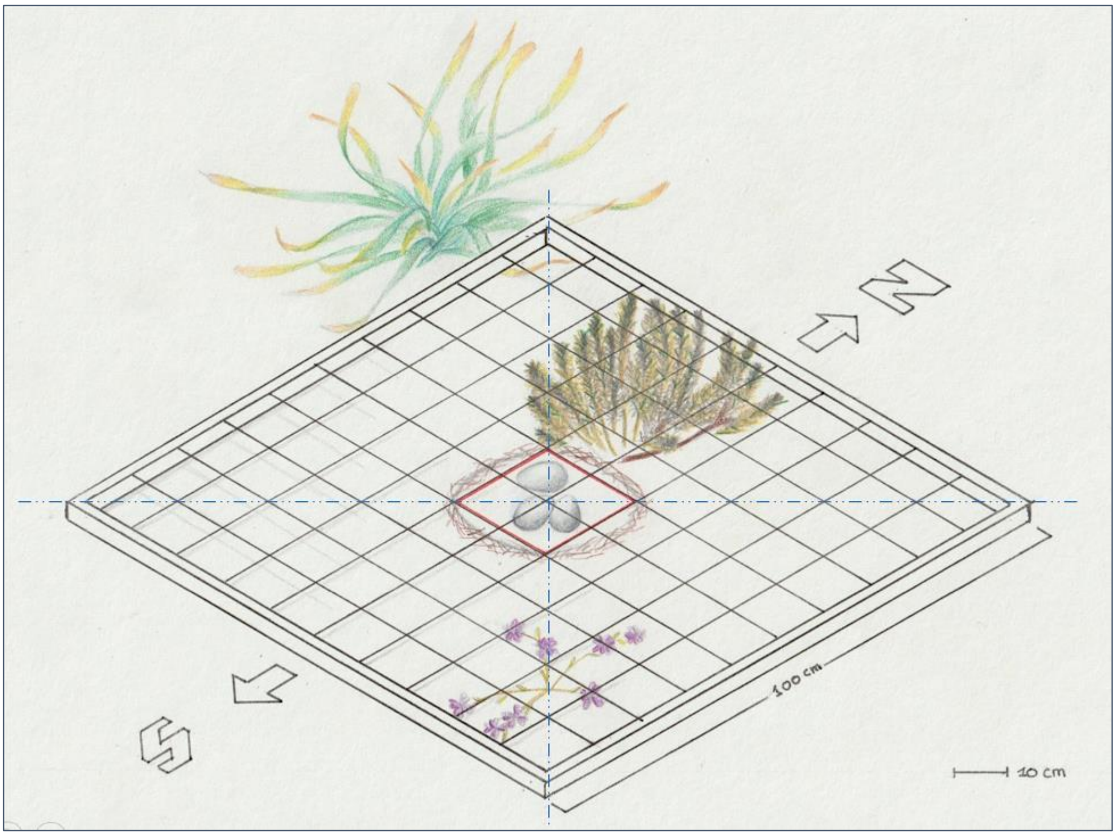

***Figure 2** Graphic representation of the quadrat used to characterize the vegetation around the nest in terms of percentage of cover and size (height), within one meter square around the nest cup (red square). The reference quadrat position was the same for all the nest with each side for a cardinal point. The quadrat was divided in four triangular dials one for each cardinal point (blue dashed lines), to assess the vertical prospect of vegetation coverage, in terms of height (in cm). The three plants represented are reproductions of in-situ vegetation examples.*

monitoring the nests, recording the eggs' measures (length and breadth) with a calliper (±0.01 mm) and taking note of the eggs' laying order (by numbering them with a soft pencil) until each marked nest reached the complete clutch.

All the 124 nests were characterized regarding their vegetation coverage using a 1 m^2 quadrat divided in a grid of 100 smaller quadrats of 10 cm. The quadrat was placed always in the same way with each side oriented to a cardinal point and centred on the nest cup (figure 2). The percentage of vegetation cover was quantified for the entire quadrat area (Nest Coverage), and for the nest cup using the four central sub-quadrats making up a total area of 40 cm^2 (Cup Coverage). We also registered the maximum height of the vegetation in each cardinal dial (MaxH West, MaxH Est, MaxH North, MaxH South) to have a vertical prospect of the coverage (figure 2). Finally, we monitored the nests to determine the number of chicks hatched in each clutch in relation to the number of eggs laid (Hatching Success = (number of chicks in the nest/ number of eggs in the nest) * 100). The control area was established to compare its hatching success with the experimental area and to assess if our experimental setup had any unwanted impact on the breeding success.

2.3.4 Experimental design

We aimed to evaluate the variation of the heart rate of the incubating parents and the time needed by the parents to resume incubation after disturbance, as proxies of the behavioural and physiological stress caused by the anthropogenic disturbance in the two gull species. We also evaluated the temperature variation of the clutch during disturbance and consequent incubation absence periods. For this we established a transect in each of the two experimental areas (one for each species) based on the virtual line of the monitoring phase, assuring the maximum possible proximity of all the marked nests to the established transect. During the second and third weeks of incubation, each nest was sampled twice (Original and Replicate data), for approximately 7h (between 9.:30 and 16:30/17:00) with a 3- day interval between the two sampling periods. We chose this period of the incubation stage in order to minimise the probability of affecting the hatching success due to nest desertion. The sampling of each nest consisted of retrieving an egg of the clutch, taking note of the measures (length and breadth) for identification and to avoid to collect the same egg during the Replicate sampling phase; replacing the egg with a heart rate and temperature minimally-invasive recording device (dummy egg; DE); deploying a thermohygrometer (TH; UNI-TREND, UT 330B USB data logger) in the nest cup border (for microhabitat temperature characterization) and a video recording camera. (Annex 1)

We went through these phases as quickly as possible to reduce the disturbance produced by the set-up phase, maintaining a regular walk path across the colony. During the data collection period, the real egg (identified per nest and size) was placed inside an incubator (Sailnovo TOYS2 incubator 40w ac 195-245v) at 38ºC and returned to the nest at the end of that day's sampling period.

After the set-up of the monitoring tools, we performed the disturbance stimuli twice a day, one in the morning (Transect 1) and one in the afternoon (Transect 2), walking through the previously established transect for 10 minutes in each of the experimental areas. When walking the transect, two persons approached the colony at a regular pace, avoiding sudden movement and maintaining a short distance between them, in order to act as one. We conducted the transect in the morning around 10:00/10:30 h (end of Period I; see *data extraction* paragraph below) and in the afternoon around 14:00/14:30 h (end of Period II), all the procedures were concluded every day around 16:30-17:00 h (end of Period III), when the monitoring equipment was retrieved and the real eggs were placed back in the nest.

We sampled a total of 8 nest each day deploying automatic cameras, one for each nest, during the entire sampling time each day (approximately from 9:30 until 17:00 h), moreover observations with binoculars were made from a portable bird-hide to complement the cameras' records (Annex 2). To avoid disturbing the birds during the observational period in the hide, only one of the observers stayed in the hide, while the second observer finished the transect and walked away from the colony. This approach was needed as our goal was to take note of the birds' behaviour in absence of anthropogenic disturbance. At the end of the afternoon observational period (Period II), the bird-hide was positioned in a different location so as to observe new nests the following day. All data collection considered the nest as a sampling unit, and no biometric characterization of the breeding gulls was made.

The tool: Dummy egg

The 48 experimental nests selected had at least 2 eggs. For each of these nests, we acquired heart rate recordings of the incubating adults and the clutch's temperature through the deployment of a total of 8 dummy eggs, twice (Original and Replicate data), in 8 different nests for approximately 7 hours each day.

The dummy egg is an electronic device based on Arduino, a hardware platform consisting of a series of electronic boards equipped with a microcontroller. The external structure of the dummy egg consists of an external plastic shell manually painted to resemble the true eggs of the species and has a middle size between the yellow-legged gull and Audouin's gull eggs. The dummy egg was featured with a microphone (located on the inner surface of the shell, under a 5mm- diameter drilled hole that was covered externally by a thin nitrile membrane to increase resonance), and a thermometer to record, respectively, the heartbeat of the incubating gull and the temperature reached by the clutch. The data was recorded in a micro-SD memory card linked to a mini-USB port allowing the data to be downloaded through a terminal application (Qt C++ toolkit, version 5.15.2). Two 3.6V lithium batteries provided the device with a record autonomy of approximately 60 hours. Finally, the dummy egg presents an on/off switch located as shown in Figure 3. The dummy egg is a contact-based sampling tool, meaning that as soon as the bird took off the signal was lost.

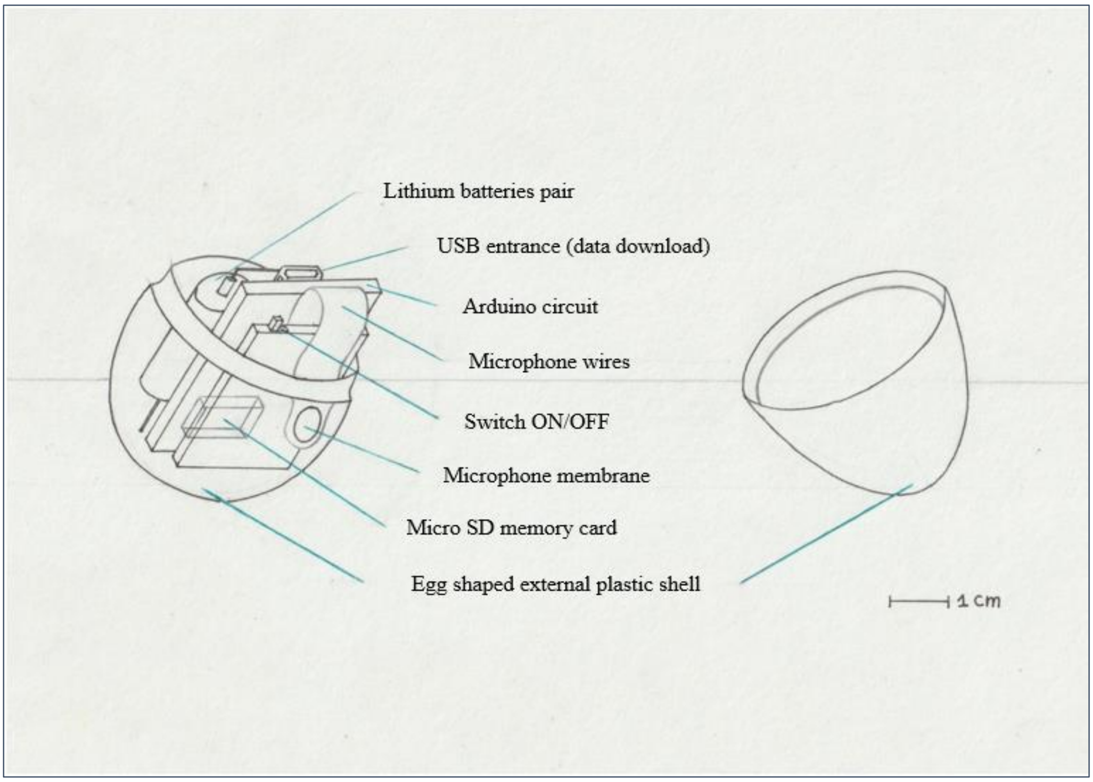

Figure 3 Graphic section of the dummy egg device's structure and components used to record the heart rate and the clutch temperature of Yellow-lagged and Audouin's gull adults and nest, during the breading season in 2021, in Deserta island, south coast of Portugal.

Analysis of heart rate recording

Each of the audio files recording the heart rate, was analysed with the support of two different software to extract data. The first software is a multi-track audio editor and recorder, named Audacity (version 3.0.0), used to manually validate the presence of heartbeat at random intervals and manually obtain the heart rate during stress conditions (Stress HR). We defined stress condition as the time period during the transect between when the nesting gull, detecting the incoming operator's presence and perceiving it as a threat, showed the physiological preparation for a "fight or flight" reaction (detected by a rise in heart rate) until the gull took off (absence of heart rate signal). Consequentially, we defined the heart rate (HR) recorded in the absence of anthropogenic stimuli as basal heart rate (Basal HR; Annex 3). The second software is a costume-written software (Laboratório de Instrumentação e Física Experimental de Partículas (LIP), Coimbra University, Portugal) which was used to obtain the baseline heart rate data (Basal HR), and is composed of one set of operations executed in sequence. This set of operations detects and counts the heartbeats in the frequency domain of the signal. The user, through the software interface, can modify the amplification signal, filter and empower the raw signal (Hann function), and find features in the power of the filtered signal (difference of Gaussians, DoG) to remove noise such as gulls' vocalizations. Once the parameters were set up and the program was run, it produced an excel file (CSV format) and a second WAV file for Audacity. This second file contained the recorded soundtrack aligned with the amplitude of the DoG track, which was important as a debug tool to confirm the heart rate given by the software analysis in the CSV. file for a given period. The CSV file contained the heart rate values extracted as 15 seconds mean of the heart rate data recorded. We used this file to extract the heart rate during the absence of disturbance (Basal HR). The same file also provides the date, the time, and the standard error for each value. All the data provided by the software was checked manually.

Data extraction

We divided each sampling day into three different time intervals (figure 4): Period I, which began with the deployment of the dummy egg (9:00-9:30 h) and finished 30 minutes after the end of the Transect 1 (around 10:00 -10.30 h); Period II, that began at the end of Period I and ended 30 minutes after the end of the Transect 2 (around 14:00 - 14.30 h); and period III, that start at the end of Period II and ended with the retrieval of the dummy eggs. For each Period (I, II, and III), of the Original and Replicate sampling days of each nest, we extracted the basal heart rate (Basal HR; 5 minutes interval), the stress heart rate (Stress HR; 1-minute interval counting back from the gull taking off during stress condition – see above), the time interval between the end of the transect and the resuming of the incubation (Resume time; absence time minus 10 minutes transect time) and the minimum and maximum temperature recorded by the dummy egg – a proxy of clutch temperature- during the transect until the gull resumed incubation (variation between the maximum and the minimum temperature; DE temperature variation_gull's absence), and during the 10 minute of the transacts (DE temperature variation_10 min transect).

Regarding the heart rate under stress conditions (Stress HR) we obtained measurements 3 times each day: immediately before the gull took off during the two transects (ST1 and ST2) and an additional measurement before the dummy egg was retrieved (S3). We considered this last condition as an additional stress event to collect an extra set of data for Stress HR, although we lack the resume time and dummy egg temperature data for this event. Every Basal HR extracted before the transect of the same Period was compared with the subsequential Stress HR obtained from the disturbing event within the same Period to assess the effect on the gulls' heart rate stress response (figure 4).

Based on Borneman et al. (2014) study, we standardized the heart rate response (SHRR) according to body mass for the yellow-legged gull and Audouin's gull to be able to compare the effects of anthropogenic disturbance between the two species. The SHRR was calculated for each period as the ((Stress HR - Basal HR/ Basal HR) *100), and divided by the mean body mass of each species (yellow-legged gull: 1000g, Matos et al., 2018; Audouin's gull: 600g, Verissimo, 2018).

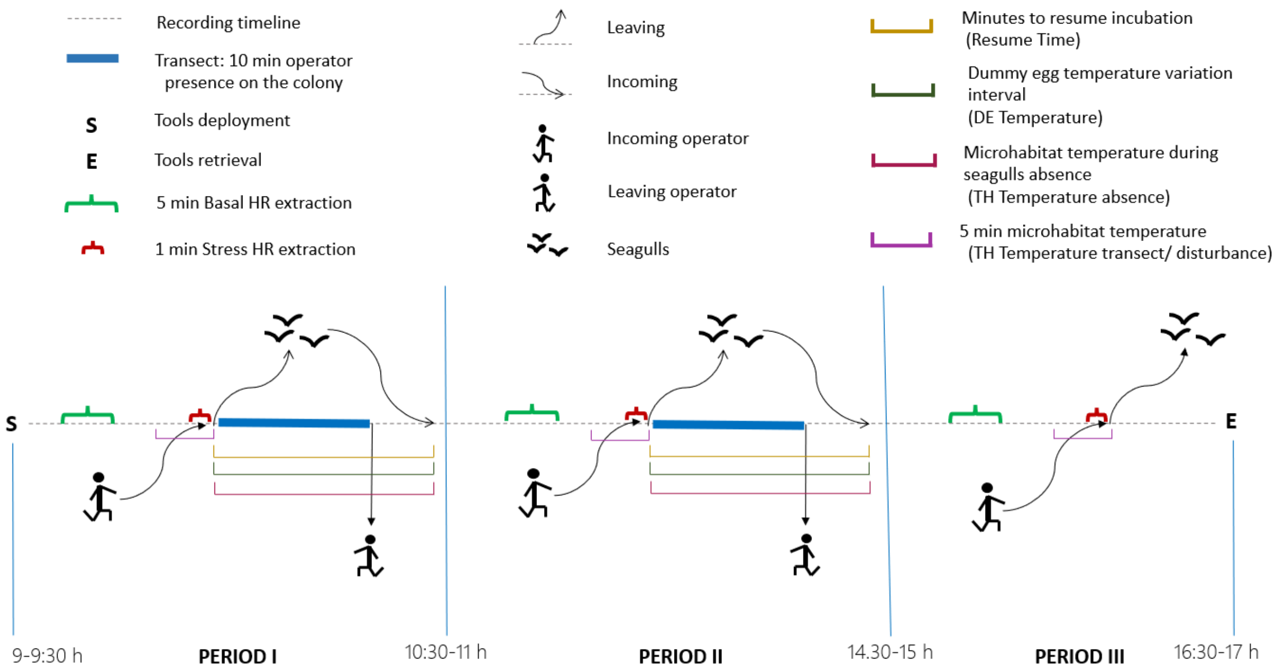

Figure 4 *Diagram of the experimental design in relation to the data extraction from the dummy egg device and the thermohygrometer used during the study of anthropogenic impact on Yellow-lagged gull and Audouin's gull, in the breading season in 2021. The procedure represents S to E stands for a day of recording. The operations reported in this diagram were repeated for three consecutive days for the Original sampling, recording 8 nests each day making up total of 24 nests for each species. Thereafter, the other three days of sampling constituted a replicate for the same nests, in the same order (Replicate).*

Regarding the thermohygrometer data we extracted a 5 minutes interval mean, before each transect, to assess the microhabitat temperature right before the gull took off (TH temperature before transect/ disturbance), and the microhabitat temperature during the 10 minutes of transect (TH temperature transect). We also extracted the mean temperature for the entire period of gull absence until it resumed incubation for each transect (TH Temperature absence), to evaluate the environmental temperature influence on the egg temperature during these intervals (figure 4).

2.3.5 Statistical analysis

We firstly checked the assumption of normal distribution, performing Shapiro-Wilk's tests, and homoscedasticity, with a Levene's test, for all data collected. The egg volume, the hatching success, the vegetation coverage, and the SHRR variable did not present a normal distribution and homogeneity of variances. Therefore, for these variables, we performed the analyses using Kruskal-Wallis non-parametric tests.

We performed a principal component analysis (PCA) to reduce the vegetation variables that were used to characterize the nests (Nest coverage, Cup coverage, MaxH West, MaxH Est, MaxH North, MaxH South) to one principal component (PC1) that explained most of the variation in these data (Annex 4). To perform this multivariate analysis the variables in percentage were first ARCSIN(x) transformed, and the maximum vegetation height was calculated as a mean of the vegetation height in each cardinal dial of the quadrat. We used the library "psych" for the PCA function and the "devtools" library for the graphic evaluation of the PCA results.

We compared the hatching success, egg volume, and vegetation characteristics (PC1) to assess possible differences between the experimental and the control nests for each species. The Hatching success and the principal component (PC1) scores resulting from the PCA did not show a normal distribution and were analysed with the non-parametric test. Regarding the egg volume as after the logarithmic (log(x)) transformation, it presented a normal distribution and homoscedasticity, a one-way ANOVA was used.

To evaluate the success of the disturbance stimuli in terms of heart rate response, we ran a paired t-test between the heart rate values before (Basal HR) and during the disturbance events (Stress HR), separately for each of the three different defined Periods, and each species. In this analysis only the nests with complete information about Basal and Stress HR were used, all the other nests were omitted to create a balanced dataset. We constructed the datasets and performed the same analysis for the data extracted in the replicate sampling days. We also analysed the effect of the anthropogenic disturbance on the heart rate response, for each species, applying a paired t-test for each of the overall sampling days between the Basal and Stress HR data, i.e., not considering the different periods of the day. Regarding the SHRR analyses, we conducted a Kruskal-Wallis test, to assess the difference in the median SHRR between yellow-legged gull and Audouin's gull. Moreover, we used a paired t-test to compare the values of the Original data with the Replicates for each species and for each Period of the day, to assess the difference between these two stages of data acquisition, and therefore the confirmation of their similarity across time, for the SHRR and Resume Time data.

In order to further assess the effect of other potential explanatory variables on the SHRR, the Resume Time, the DE Temperature, and the DE Temperature during 10 minutes we ran a Generalised Linear Mixed Model (GLMM) for each of these dependent variables, and a Generalized Linear Model (GLM) for the Hatching Success. We constructed the models using the packages "lme4" in R studio and evaluated their correctness and power respectively through the study of the normality of the residuals and the Akaike's Information Criterion (AICc). We used the Species, the Period, the PC1, and the TH Temperature, as fixed variables for all the models. Because each nest was sampled twice, we used the nominal variable Original/Replicate sampling (O/R) nested within Nest ID as a random variable, to control for the non-independence of samples in the GLMM models.

For the SHRR and the Resume Time models, we used the TH Temperature extracted as the 5 minutes mean of values right before the gull take off (TH temperature before transect/ disturbance). Regarding the DE Temperature model, we select the TH Temperature absence, the mean thermohygrometer temperature recorded during the absence time of the gull under disturbance. Finally for the DE temperature during the 10 minutes model we used as microhabitat temperature the mean of values extracted during the 10 minutes of transect (TH temperature transect), in addition to Species and PC1 explanatory variables.

The GLM on the Hatching Success was selected using the AIC's ranking methodology using as explanatory variables the DE Temperature, the TH Temperature absence, the Resume Time, and the PC1 (Annex 7).

All the analyses were performed using the program R and run within R Studio (version 1.4.1717, R Core Team 2019) with an alpha threshold of 0.05.

2.4 Results

2.4.1 Overall characterizations of nest vegetation and breeding parameters

A total of 62 nests for each species was used (24 for the experimental area; 38 for the control area) to evaluate vegetation characteristics of the nest, the clutch size, and egg volume of each clutch and to assess if there was any influence of the experimental design on the hatching success.

Principal Component Analysis of vegetation microhabitat

In the principal component analysis (PCA) run on the vegetation variables collected (Nest coverage; Cup coverage; MaxH mean of each of the four cardinal points), 2 axes were extracted (Annex 4): PC1 explaining 59.31 % of the variation and PC2 explaining the 11% of the variation. For the following statistical analyses, we used PC1 scores as a proxy for the nest vegetation cover, as it explains more than the 50% of the results.

Regarding the nests of yellow-legged gull the characteristics of the vegetation (PC1) in the two areas (control versus experimental) appear comparable ($\chi^2_{1,62}$ = 0.079, p = 0.778). However, the mean difference in vegetation coverage between control and experimental nests of Audouin's gull shows a p-value under the threshold (< 0.05), indicating the presence of statistically significant differences between the two areas in terms of vegetation ($\chi^2_{1,62}$ = 18.549, p < 0.001; Table 1).

Table 1 *Nest vegetation cover and main breeding parameters of the incubating gulls' nests in the experimental (subjected to planned disturbance by the researcher) and control (without planned disturbance by the researcher) areas. The percentage of vegetation coverage (PC1) was obtained from a Principal Component Analysis. Egg volume variable data have been LOG(x) transformed to obtain a normal distribution.* Statistically significant differences (*p<0.05*) *for one-way ANOVA (Egg Volume) and Kruskal-Wallis tests (Hatching success and PC1) between experimental and control groups within each species, are in bold.*

	Larus michahellis				*Ichthyaetus audouinii*			
	Experimental		**Control**		**Experimental**		**Control**	
Variable	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n
PC1	0.08 ± 1.7	24	-0.11 ± 1.	38	**1.66 ± 2.2**	**24**	**-0.98 ± 2**	38
Hatching success (%)	0.47 ± 0.4	24	0.31 ± 0.4	38	**0.48 ± 0.4**	**24**	**0.8 ± 0.3**	38
Egg volume (mm3)	85.48 ± 8.2	24	82.83 ±7.6	38	65.36 ± 3.1	24	66.75 ± 4.8	38

Egg size and Hatching success

The mean egg size in each clutch did not differ significantly between the control and experimental areas of yellow-legged gull ($F_{1,62}$ = 1.616, p = 0.209) and of Audouin's gull ($F_{1,62}$ = 1.442, p = 0.235; Table 1).

Regarding the hatching success, there were no significant differences between the control and experimental areas of yellow-legged gull ($\chi^2_{1,62}$=1.91, p = 0.167; Table 3). On the other hand, the hatching success was significantly different between the two areas of Audouin's gull, with the control area that had the 0.80 % hatching success over the 0.48 % of the experimental area ($\chi^2_{1,62}$=11.36, p = 0.001; Table 1).

2.4.2 Anthropogenic disturbance effect and vegetation influence on incubating gull's heart rate, incubation behaviour, clutch temperature and hatching success

We collected a total of 288 intervals of 5 minutes each (144 for yellow-legged gull and 144 for

Audouin's gull) to evaluate the resting baseline heart rate (Basal HR) and 288 intervals of 1 minute for the heart rate under stress conditions provided by the walking-operator's disturbance (Stress HR).

Yellow-legged gull (*Larus michahellis*)

The results show a significant increase in the heart rate in the minute right before the bird took-off when compared with the baseline record, collected during the incubation time in absence of disturbance (for procedures intervals see Figure 4).

In particular, in yellow-legged gull, such an increase was observable at all sampling Periods throughout the day during the Original sampling (Period I: t_{19}=-2.846, $p = 0.01$; Period II: t_{18}=-4.638, $p < 0.001$; Period III: t_{12}=-2.766, $p = 0.017$). The heart rate increase was maximum in the Period I with a range that went from 148.9 ± 40 to 188.9 ± 48 heartbeat/min. In the Replicate sampling, there was a significant increase in HR in Period II and III (t_{17} = -5.943, $p < 0.001$; t_{19}=-3.063, $p < 0.05$, respectively), while in the Period I the Stress HR was not significantly higher than the Basal HR (t_{14} = -1.810, $p = 0.092$). We detected the maximum variation in Period II with 127.6 ± 20.6 and 170.0 ± 33.1, respectively as baseline and stressed heart rate values (Table 2).

Overall considering the entire day time, for yellow-legged gull, both in the Original and Replicate sampling, there was a significant increase in heart rate during disturbance (t_{51} = -5.5, $p < 0.001$; t_{52} = 5.805, $p < 0.001$, respectively; Table 2).

Audouin's gull (*Ichthyaetus audouinii*)

In Audouin's gull the results of the Original sampling, regarding the increase of heart rate during the transect, show a significant difference between Basal and Stress HR for Period II and III (t_{15} = -4.356, $p < 0.001$; t_{17} = -2.682, $p < 0.05$, respectively). In Period I there were no significant differences (t_{15} = -1.934 and $p = 0.072$) as reported in Table 2.

The paired t-test results for the Replicates sampling dataset show a significant difference between Basal and Stress HR only for Period III (t_{14} =-3.895, $p < 0.002$) with 133.8 ± 21 heartbeat/min for baseline and 153.3± 14.3 for the stress under disturbance circumstance. Periods I and II did not show any significant difference across the two circumstances (Period I t_{15}= -1.795, $p = 0.093$; Period II t_{10}= -0.073, $p = 0.943$).

Table 2 *Mean (±SD) heart rate measurements of yellow-legged gull (Larus michahellis) and Audouini's gull (Ichthyaetus audouinii) during the absence of disturbance periods (Basal HR) and disturbance experiment events (Stress HR). The disturbance was performed during three distinct periods of the day: Period I, which began with the deployment of the dummy egg (9:00-9:30 h) and finished 30 minutes after the end of the Transect 1 (around 10:00 -10.30 h); Period II, that began at the end of Period I and ended 30 minutes after the end of the Transect 2 (around 14:00 - 14.30 h); and period III, that start at the end of Period II and ended with the retrieval of the dummy eggs (around 16:30/17; see data extraction paragraph in material and methods). Original and replicates refer to two different phases of data collection obtained from the same nest after three-day intervals. Statistically significant values (p<0.05) for paired t-tests between Basal and Stress HR within each species and sampling period (or "all periods") are in bold.*

		Larus michahellis			*Ichthyaetus audouinii*		
	Period of day	**Basal HR (heart beat/min)** Mean ± SD	**Stress HR (heart beat/min)** Mean ± SD	N	**Basal HR (heart beat/min)** Mean ± SD	**Stress HR (heart beat/min)** Mean ± SD	N
Original	*Period I*	**148.9 ± 40**	**188.9 ± 48.0**	20	154.9 ± 21.4	167.9 ± 30.6	16
	Period II	**146.3 ± 28.1**	**182.9 ± 25.7**	19	**142.6 ± 14.9**	**164.9 ± 25.8**	16
	Period III	**143.4 ± 36.5**	**173.6 ± 22.4**	13	**147.1 ± 28.1**	**168.6 ± 31.6**	18
	all Periods	**146.6 ± 35.1**	**182.9 ± 35.9**	52	**148.1 ± 22.9**	**167.2 ± 29.6**	50
Replicate	*Period I*	139.7 ± 33.4	155.3 ± 20.4	15	149.2 ± 22.7	162 ± 20.2	16
	Period II	**127.6 ± 20.6**	**170.0 ± 33.1**	18	137.9 ± 18.5	138.2 ± 18.0	11
	Period III	**134.9 ± 24.1**	**166.25 ±40.8**	20	**133.8 ± 21.**	**153.3± 14.3**	15
	all Periods	**133.8 ± 26.5**	**164.4 ± 34.0**	53	**140.7 ± 22.4**	**152.6 ± 20.1**	42

However, as for yellow-legged gull, the evaluation of the three Periods together, whole day, shows a significant difference between heart rate with and without disturbance for both Original and Replicate sampling data (t_{49} = -4.851, P < 0.001; t_{41} =-3.367, P < 0.002 respectively; Table 2). Figure 5 graphically shows the variations between undisturbed and disturbed heart rate response across the three Periods of the day, in both species separately.

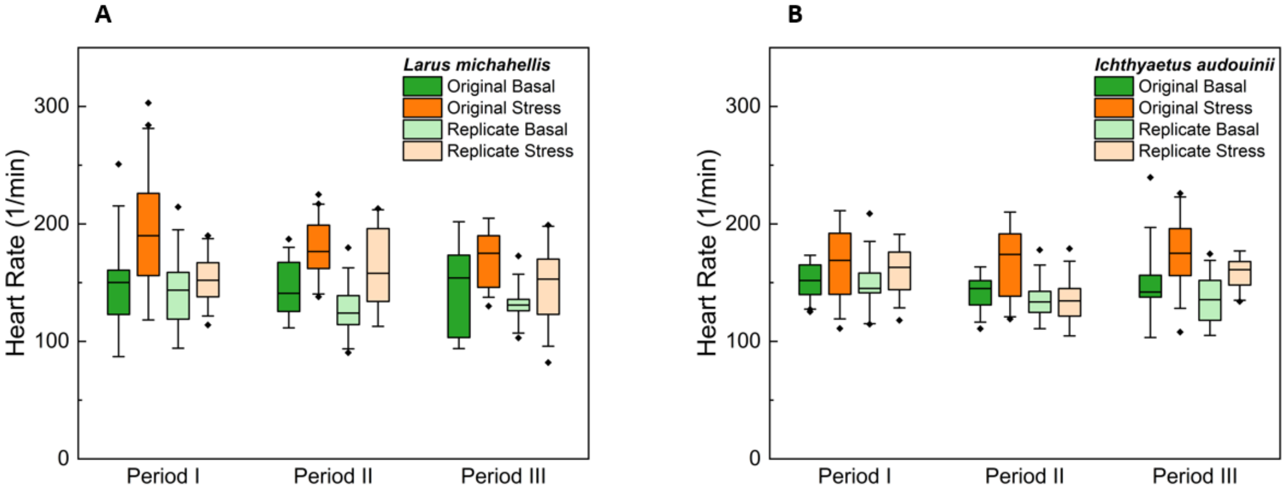

Figure 5 *Heart rate (1/ minutes) variation in the yellow-legged gull (Panel A) and Audouin's gull (Panel B), under undisturbed circumstances (Green box) and under experimental disturbance (Orange boxes). The measures are reported both for original (dark colours) and replicate (light colours) samples from 24 analysed nests. Original and replicates refers to two different phases of data collection obtained from the same nest at a three-day interval The three periods of the day represent respectively the time since the equipment deployment until the end of the first disturbance event (Period I: 10:00 -10.30 h), until the end of the second event of disturbance (Period II: 14:00 - 14.30 h) and until the equipment withdrawal (Period III: 16:30 – 17:00 h). Standard deviation is represented at 1.5, and confident interval between 25%-75%. The rhomboid dots indicate outliers.*

Heart rate variation and incubating behaviour correlate across species

The comparison of the Standardized Heart Rate Response (SHRR; Annex 9) between the two species, across each Period of the day (I, II, and III) unexpectedly showed mostly non-significant differences (Table 3). The rate of change in heartbeat of yellow-legged gull and Audouin's gull in response to anthropogenic disturbance, in all the periods of the original sampling data ($\chi^2_{1,38}$= 0.228, p = 0.633; $\chi^2_{1,35}$= 0.004, p = 0.9472; $\chi^2_{1,32}$= 0.369, p = 0.543) does not differ significantly, despite the values take in account the relative weight of each species. Regarding the Replicate sampling data, there was also no significance for Period I and III ($\chi^2_{1,31}$= 0.001, p = 0.9835; $\chi^2_{1,35}$= 0.565, p = 0.452, respectively). However, there were significant differences in the SHRR between species in Period II ($\chi^2_{1,29}$= 10.945, $p < 0.001$) of the Replicate sampling, with Audouin's gull showing a smaller HR increase than yellow-legged gull.

Using GLMM to evaluate the effect of other potential explanatory variables (Annex 5), apart from bird species, on the standardized heart rate response (SHRR), we found that it was not influenced neither by the specie nor the time of the day (Period I, II and III) or the vegetation coverage (Table 4).

Table 3 *Mean (±SD) of the change of HR between Basal HR and Stress HR, in yellow-legged gull (Laurus michahellis) and Audouin's gull (Ichthyaetus audouinii). The data were normalized using the mean body size of each species (see text). To distinguish the data coming from different disturbance events we divided the day into three periods: Period I, which began with the deployment of the dummy egg (9:00-9:30 h) and finished 30 minutes after the end of the Transect 1 (around 10:00 -10.30 h); Period II, that began at the end of Period I and ended 30 minutes after the end of the Transect 2 (around 14:00 - 14.30 h); and period III, that start at the end of Period II and ended with the retrieval of the dummy eggs (around 16:30/17; see data extraction paragraph in material and methods). Original and replicates refers to two difference phases of data collection obtained from the same nest at three-day intervals.* Statistically significant values (*p<0.05*) *for Kruskal-Wallis test for SHRR between species are in bolt*

		Change in HR (SHRR)			
		Larus michahellis (heart beat/min)		*Ichthyaetus audouinii (heart beat/min)*	
		Mean ± SD	N	Mean ± SD	N
Original Time of day	Period I	29.10 ± 56.05	14	15.86 ± 24.34	14
	Period II	30.54 ± 32.11	14	30.48 ± 20.98	14
	Period III	18.71 ± 45.86	14	29.41 ± 36.87	14
Replicate Time of day					
	Period I	19.84 ± 29.68	10	19.18 ± 21.87	10
	Period II	**37.32 ± 24.79**	10	**4.36 ± 14.70**	10
	Period III	20.90 ± 33.81	10	29.15 ± 30.32	10

However, SHRR seems to be slightly affected (although non-significantly, p = 0.05) by the microhabitat temperature (TH temperature transect/disturbance) right before disturbance; the positive estimate reflects a positive relationship between SHRR and TH temperature transect/disturbance, meaning that at higher temperature there is a tendency for a higher magnitude of heart rate increase. Regarding this last evidence, a supplementary GLM model has been runed using Basal HR as dependent variable and the TH temperature during the relative intervals of extraction, along with the Species and the time of the day (Period I, II and III) as explanatory variables (Annex 6). The model so runed, shows that an inverse relationship between Basal HR and TH temperature, assessing for lower Basal HR with higher values of microhabitat external temperature. This model moreover, shows that the Period III of the day record for the lowest Basal HR values. The GLM model on Resume Time revealed that time to resume incubation differed between transects within in each day, with Transect 2 (p < 0.001) showing a shorter resume time than Transect 1 (Table 5), in both species (Figure 6).

Table 4 *Results of the generalised linear mixed models (GLMM) on the variation of the standardized change of the heart rate between baseline and disturbance periods (standardized heart rate response - SHRR; See the formula in the text) in the yellow-legged gull (YLG) and Audouin's gull (AG). Periods II and III are two of the three periods of the day that represent, respectively, the time: from the equipment deployment until 30 minutes after the end of the first disturbance event (Period I: 10:00 -10.30 h), until 30 minutes after the end of the second event of disturbance (Period II: 14:00 - 14.30 h). The percentage of vegetation coverage (PC1) was obtained from a Principal Component Analysis. TH Temperature refers to the nest microhabitat temperature recorded by the thermohygrometer. The number in bold identifies a significant effect (p < 0.05).*

		SHRR			
		Estimate	t	p	Main effects
Species (YLG/AG)		0.013	1.525	0.798	_
Day Time	Period II	0.004	-0.766	0.671	_
	Period III	0.006	1.142		
PC1		0.002	1.595	2.648	_
TH Temperature (°C)		**0.001**	**0.561**	**0.055**	The higher the temperature the greater the SHRR

Moreover, the Resume Time differed between species (p < 0.05): yellow-legged gull appears to take longer to resume incubation than Audouin's gull (Table 5; Figure 7). Finally, the microhabitat temperature (TH Temperature transect/disturbance) shows a negative relationship with the Resume Time, suggesting a shorter absence from incubation duties with higher temperatures.

Regarding the variation of the clutch temperature during the absence of the incubating gulls (DE Temperature) we found that the only variable affecting it was the gull species (p < 0.05). The temperature of the clutches (measured by the dummy egg) of yellow-legged gull showed a lower variation when compared to Audouin's gull. Despite this, yellow-legged gull showed a longer time to resume incubation than Audouin's gull (Table 5).

The GLMM conducted in the attempt to explain this apparent countersense, on the temperature of the clutch, recorded by the dummy egg, within the bare 10 minutes of the walking-operator's disturbance Transect, does not show to be influenced by any of the variables accounted for.

Table 5 *Results of the generalised linear mixed models (GLMM) on the variation of resume time (the time from the disturbance until the gull incubation recovery; Resume Time, min), on the variation of the clutch's temperature during adult absence from the nest (DE Temperature variation gull's absence, ºC), and the variation of temperature in the 10 minutes of transect coinciding with gulls' absence from nest (DE Temperatures variation 10 min Transect; °C). Species represent the two analysed model species: the Yellow-lagged gull (YLG) and the Audouin's gull (AG). Transect 1 and 2 represent the two walking-operator's disturbance events, respectively around 10:00 -10.30 h and 14:00 - 14.30 h. The percentage of vegetation coverage (PC1) was obtained from a Principal Component Analysis. The TH temperature (°C) stands for nest microhabitat temperature recorded by the thermohygrometer as a mean of the 5 minutes before the gull's take-off, as a interval mean during gull's absence and as a interval mean during the 10 minute Transect respectively for Resume time model, and DE temperature variation gull's absence and DE temperature variation 10 minutes transect models. The number in bold identify significant effect.*

	Resume Time				**DE Temperature variation gull' s absence**				**DE temperature variation 10 min transect**			
	estimate	t	p	Main effects	estimate	t	p	Main effects	estimate	t	p	Main effects
Species (YLG/AG)	0.3410	2.443	**0.015**	Yellow-lagged gull > Audouin' s	-0.5958	-4.063	< **0.001**	yellow-legged gull < Audouin' s gull	-0.3076	-0.519	0.603	_
Transect (1/2)	-0.183	-3.881	< **0.001**	Transect 2 < Transect 1	-0.051	-0.864	0.388	_	-0.016	-0.057	0.954	_
PC1	0.011	0.396	0.692	_	0.020	0.746	0.456	_	0.143	1.387	0.165	_
TH Temperature (°C)	-0.017	-1.972	**0.049**	The lower the temperature the longer the resume	-0.009	-0.812	0.417	_	-0.080	-1.632	0.103	_
Resume Time (Min)	_	_	_		0.0234	1.289	0.197	_	-0.126	-1.752	0.080	_

Table 6 *Results of the generalised linear model (GLM) on the hatching success (HS; See the formula in the text). PC1 state for the nest vegetation characterization derived from the Multiple Components Analysis. Resume time (minutes) is the time occurring between the nest abandon until the gull's incubation resume, after the walking-operator's disturbance event. The number in bold identifies the significant effect.*

	Hatching Success			
	estimate	t	p	Main effects
PC1	4.592	2.972	**0.003**	The higher the vegetation the higher the hatching success
Resume Time (Min)	-1.725	-1.211	0.228	_

The GLM on hatching success (Annex 7) revealed that the hatching success is higher when the vegetation coverage (PC1) is higher (Figure 8), revealing a close influence of these parameters on each other ($p < 0.003$). Species, TH Temperature, Resume Time, and DE Temperature show to have no-impact on the Hatching Success variability of the sampled nests (Table 6).

To assure that the values extracted from the original and replicates sampling data, regarding the Resume Time, were statistically equivalate we run a series of paired t-tests. We analysed separately in each species, the values of the Transects' Original data against the respective data obtain during the Replicates Transects (Annex 8).

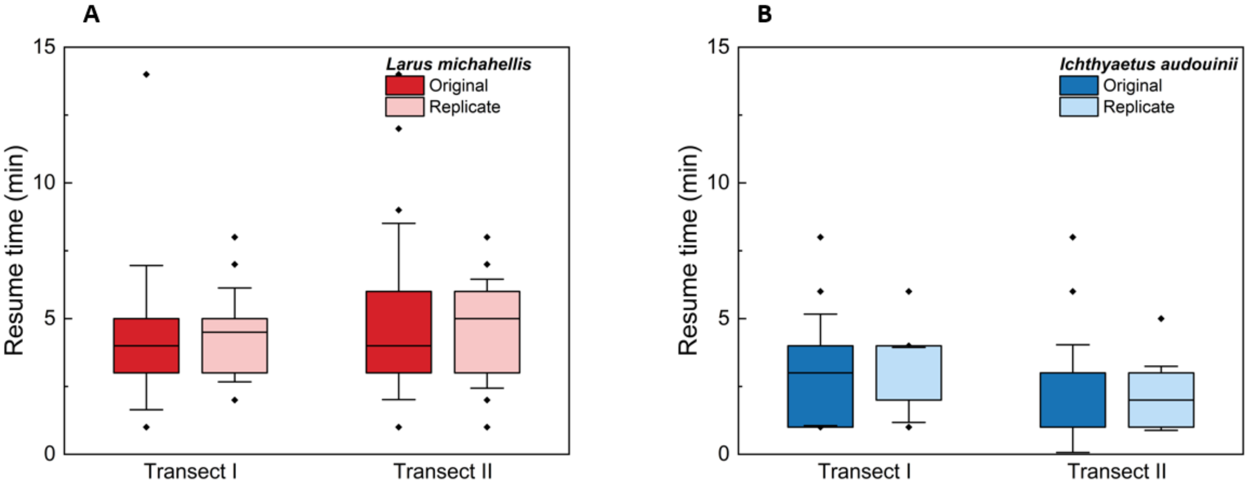

Figure 6 *Resume time (minutes) of yellow-legged gull (Panel A) and Audouin's gull (Panel B); i.e., the time that elapsed between taking-off from the nest as a response to walking-operator's experimental disturbance and the resuming of incubation. The data are extracted in minutes according to the absence of signal recorded in the dummy egg files during the two transect of disturbance event (10:00 -10:30 h, Period I; 14:00-14:30 h, Period II). The two boxes for each period represent the data sampled for the 24-nest during the Original (dark red) and the Replicate (light red) sampling phases. Original and replicates refers to two difference phases of data collection obtained from the same nest at three-day intervals. The rhomboid dots represent outliers.*

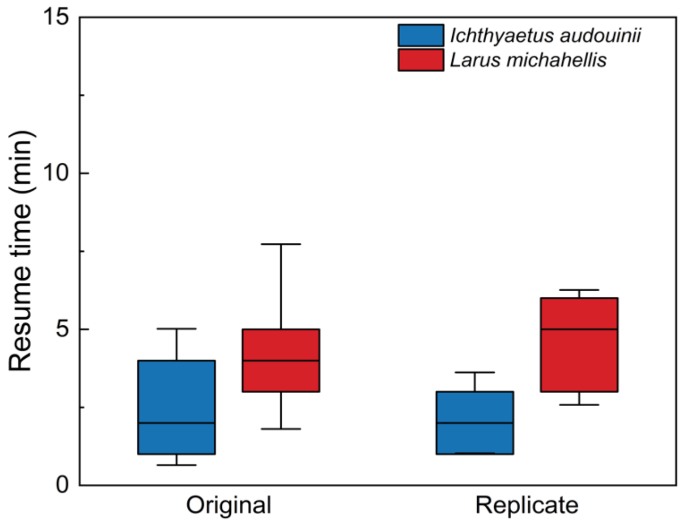

Figure 7 *Resume time (minutes), the time occurring between the nest abandonment until the incubation resume after the walking-operator's disturbance. The data correspond to all the sampled nest during the experimental time (both Transects), of both Yellow -legged gull (red boxes) and Audouin's gull (blue boxes) grouped by Original and Replicate measurements. Original and replicates refers to two difference phases of data collection obtained from the same nest at three-day intervals.*

None of the tests gave a significant p-value assessing for homogeneity between the Resume Time values during the Original and the Replicates sampling days. These results give no sign of behavioural changes in none of the two species, showing absence of habituation to the anthropogenic disturbance under the experimental conditions, during the time the disturbance was operated.

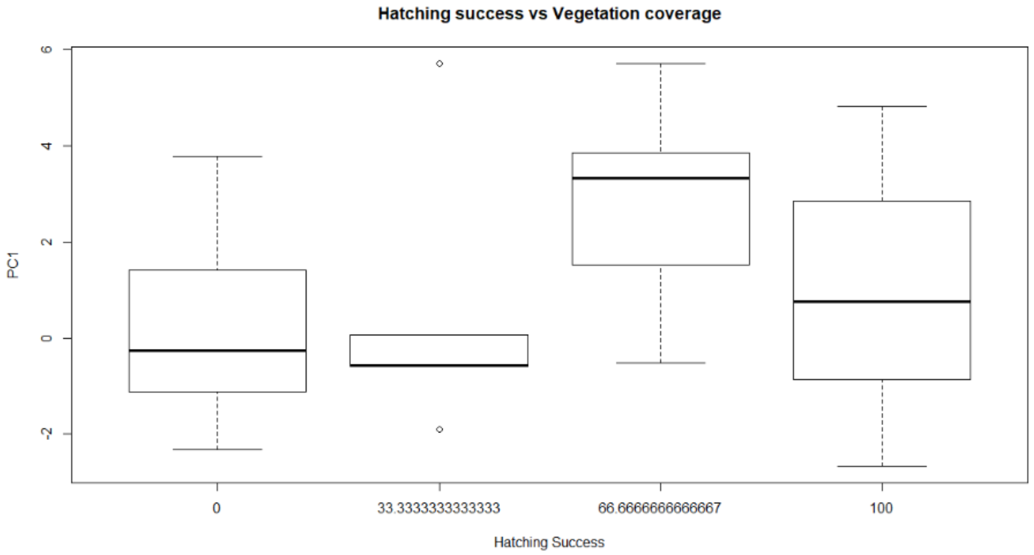

Figure 8 *Boxplot showing the distribution of the hatching success data (X axes) in relation with nest vegetation characteristic values (PC1; Y axis) derived from the Multiple Components Analysis. The data derives from the two different areas of both yellow-legged gull and Audouin's gull species (four total areas), sampled during the reproductive season 2021 in Deserta Island, south coast of Portugal.*

2.5 Discussion

2.5.1 Heart rate response to experimental anthropogenic disturbance stimuli by yellow-legged gull and Audouin's gull

This study wanted to evaluate the behavioural and physiological effects of an experimental anthropogenic disturbance on two sympatric species of gulls during the reproductive season. We found that our experimental disturbance leads the nesting adults to temporary flight-off the nest just after a sudden increase of the heart rate. The mean heart rate differed significantly between non-disturbed periods and during disturbance for both species of gulls. This confirms that anthropogenic presence in the breeding colonies is a source of physiological and behavioural stress. Humans crossing the reproductive colonies with regular path, like other incursions, are perceived as threats, triggering the "fight or flight" reaction in incubating adults, and causing parents to expend energy and leave the nests unattended (Wingfield & Sapolsky, 2003).

The magnitude of the impact of this reaction, is hypothesised to be related with the species characteristics in terms of adaptation capacity and behavioural plasticity (Weimerskirch et al., 2002; Ellenberg et al., 2008). We were expecting, therefore, to find a difference between our two model species in the range of variation of the heart rate from non-disturbed to disturbed circumstances. However, there were no significant differences in this variation between species, suggesting a physiological reaction to human disturbance comparable in yellow-legged gull and Audouin's gull. This result suggests that the different adaptation and plasticity of these two seagulls observable in some behavioural traits is not associated with different physiological response to direct human disturbance during breeding in natural colonies, at least in a short-term exposure. Therefore, regardless of the differences in behavioural plasticity and adaptation during the non-incubation period, both gulls' reproductive season can be threatened by human presence in the breeding colonies. This could be especially worrying in a scenario where suitable conditions and natural nesting sites are decreasing in availability due to anthropogenic niche expansion (Boivin et all., 2016; Haddad et al 2015). The lack of differences across the two species regarding the standardized heart rate response (SHRR) need further confirmation, as our research was conducted in a specific and limited interval of the breeding season. Also, the comparison within the same species (e.g., yellow-legged gull) in contrasting habitats (urban versus natural) would be useful.

Research conducted with similar methods -heart rate telemetry-, on Wandering Albatrosses (Weimerskirch et al. 2002; basal heart rate: 56.9±11.4 beats per minute on the nest), Adelie Penguins (Culik et al. 1989; basal heart rate: 86.0 beats per minute on the nest) and Ring-billed gull (Stout &

Schwab, 1980; basal heart rate: 198.0 beats per minute on the nest) reports different resting heart rate compared to our results (Table 2; results). It could suggest that different body mass and specific habitat temperature could partially justify the differences in the resting average HR. For instance, the average resting HR of our two species greatly diverge from Wandering Albatrosses and Adelie Penguins which inhabit colder latitudes, moreover, Wandering Albatrosses that report the lowest resting HR average also registered the highest body mass across the considered species. However, considering that Ring-billed gull, which is the closer species to the once of our study and inhabit similar latitudes, still consistently differs and, therefore, the species seems to remain the main driver influencing resting HR values. These studies also report range of variation between those species in response to human disturbance however, the conditions and the disturbance stimuli operated highly diverge from our condition to do worth it comparison. Research evaluating heart rate response to stress in gulls is limited and to our knowledge no one have took in consideration sympatric species.

2.5.2 Behavioural response to experimental anthropogenic disturbance stimuli by yellow-legged gull and Audouin's gull

Regarding the behavioural response of time to resume incubation, evaluated as the time taken by the gulls to restart incubation after they took-off under anthropogenic disturbance, the yellow-legged gull and Audouin's gull showed different responses. Contrary to our expectations, the yellow-legged gull took more time to recover incubation than the Audouin's gull (figure 2). However, the temperature variation of the clutch during the gull's absence was greater for the Audouin's gull. Because there were non-significant differences of clutch temperature variation between the two species, when considering the time interval corresponding to just the 10 minutes of the transect, we feel confident in excluding the external environmental conditions as the reason that could justify the temperature divergence between species. Therefore, we hypothesize a behavioural difference as the cause for the variation in clutch temperature to be higher in the Audouin's gull, even though it returned to incubate faster than the yellow-legged gull. We suggest that the yellow-legged gull returns faster to the nest then Audouin's gull, possibly shading the eggs but not actually resuming incubation - therefore not contacting directly with the dummy egg preventing heartbeat sound data collection - potentially adopting a looking out behaviour and simultaneously protecting the eggs from the sun. This hypothesis would justify the longer time of resuming actual incubation recorded in our results for the Yellow-lagged gull and provides a reasonable explanation for the reduced variation of the clutch temperature for this species compared to the Audouin's gull.

We suggest that this behaviour could be an adaptation of the yellow-legged gull to protect the eggs but maintaining an alert attitude to be ready for a further danger, reducing energy expenditure. However, the validation of this hypothesis would require additional data such as more detailed nest video recording analyses.

Another explanation is that this was due to the nest spatial distribution that differed between the colonies of the two species and could affect the resume time. The nests of the yellow-legged gull were more dispersed than those of the Audouin’s gull, presumably due to intraspecific competition, and, therefore the need to cover a longer distance transect for the yellow-legged gull experimental area. When the disturbance was performed in the Audouin’s colony, camera records (personal obs.), confirmed by the dummy egg records, showed that this species spent the entire time of the disturbance transect either flying in circle on top of the colony or landing distant enough from the intruder, and therefore far apart from the nests. In contrast, the yellow-legged gulls were landing back close to the nest and taking-off repeatedly during the transect. This could have led to a rising level of stress that brought the yellow-legged gulls to take longer to resume actual incubation once the disturbance stimulus was gone. The two hypotheses are not mutually exclusive and both mechanisms could be acting simultaneously but further, and more specific research is needed to understand this response e.g., comparison with the response observed under natural stimulus.

Again, regarding the time required to resume incubation after disturbance, we found that both species reduced the time needed after the second human disturbance stimulus in the same day (Transect 2). The same model that highlights this time reduction after the second disturbance of the day also showed a relation between time to resume and external temperature (Table 5). Therefore, we justify the faster recovery of incubation during the Transect 2 to be due the higher external temperature, as Transect 2 was operated in warmer hours (around 14:00 - 14.30 h) than Transect 1 (around 10:00 - 10.30 h). This could be driven by the need perceived by the gulls to protect the clutch from thermal stress. Additionally, this response could be a sign of potential habituation for both the yellow-legged gull and Audouin’s gull. Habituation, in fact, is reported to occur in response to human disturbance in many studies on colonial breeding seabirds (Borneman et al., 2014; Nisbet, 2000), particularly in situations of high frequency and intensity of disturbance (Gyuris, 2004). However, this attenuation of response that occurred within the same day was not detected between the two experimental phases - original and replicate sampling days (3 days apart) - as the resume time did not differ significantly between original and replicate samplings in the same species.

This apparent short-term habituation, but its absence in a slightly longer term, could be potentially justified by habituation occurring in gulls for events that repeat themselves within hours, but not when the disturbing events occur spaced for longer periods than half a day.

Therefore, and according to Nisbet (2000), it is more appropriate to classify the waning of response occurring throughout the events of the same day as a tolerance rather than a habituation - as it does not persist across days. We conclude that our experimental methodology provides a high frequency and intensity disturbance but for a very short time-period. In the comparative analysis between the stress heart rate recorded during the first sampling phase and the second, no differences were detected within each species. This result is in line with the generally accepted hypothesis that physiological responses, if they wane, they do so after a longer time of exposure than that required for behavioural responses to change (Culik et al., 1990; Fowler 1999). Assesses the capacity of acclimatation in Yellow-lagged gull and Audouin's gull and therefore the potential for actual habituation is, however essential, as for many other species we aim to conserve. Conservation management plans could indeed, as previously shown in other studies on different species (Fowler 1999, Lord et al., 2001; Ellenberg et al., 2008), induce habituation and avoid the damaging effects of disturbance.

2.5.3 The influence of the nest microhabitat characteristics on physiological, behavioural and reproductive parameters

The external factors such as the microhabitat environmental temperatures and the nest vegetation cover proved to be related with the physiological heart rate response, the behavioural response (time to resume incubation) and the hatching success in both species.

The variation of heart rate between disturbed and undisturbed circumstances (standardized heart rate response – SHRR), of incubating yellow-legged gull and the Audouin's gull, exhibited a positive relationship with the microhabitat air temperature that led us to further statistical investigation. We hypothesised this result to be an outcome of a general HR tendency to decrease during periods of high environmental temperature, as reported for instance in wandering albatrosses (*Diomedea exulans*) in a study by Weimerskirch et al., (2002). In their study, although performed in polar habitats, the heart rate of the incubating bird proved to be inversely related to habitat temperature, increasing when the chill-wind was blowing reducing the body temperature. Linek at al. (2021), in a study conduct on Eurasian blackbirds (*Turdus merula*) in the southwest Germany, also found a negative correlation between the recorded HR and the habitat temperature, both through the day and across seasons, with higher heart rate related to colder temperature. To check this, we tested the relationship between the Basal HR with the external temperature during the same interval. We found out that there is a slight inverse relation between the Basal HR and the external temperature (Annex 6). This same model showed that Basal HR was lower in the Period III of the day, which is approximately from 15:00 to 17:00 h. Therefore, it is possible that because the Basal HR is lower with higher temperatures, the increment of heart rate is higher during stress events in warmer habitat temperatures.

The external microhabitat temperature affected not only the physiology but also modified a voluntary reaction of both species by changing their behaviour. The yellow-legged gull and the Audouin’s gull showed a greater urgency to resume incubation when temperatures were higher. This is not a surprising behaviour as eggs, but also preliminary stages of chicks’ development are especially sensitive to high temperatures (Oswald and Arnold, 2012). Other studies that have analysed the behavioural response of seabirds to anthropogenic disturbance have also identified a relationship between temperature and nest incubation behaviour. Brown and Brown (2004) showed that Crowned Lapwings (*Vanellus coronatus*) defensive reaction decreased with higher temperatures, persisting in incubation and keeping eggs cool instead of flying away. Malayan plovers (*Charadrius peronii*), in a study by Yasué and Dearden in 2006, showed faster return to the nests that were affected by higher egg temperature, relative to ambient temperature. Therefore, it is possible to conclude that both the yellow-legged gull and the Audouin’s gull under unsuitable temperature for the clutch, actively modify their behaviour to speed up their return to the nest despite the danger perception caused by the human disturbance event.

The characteristics of the vegetation that surrounded the nests, in terms of coverage, had an important role on the reproductive success of both the yellow-legged gull and the Audouin’s gull. For both species, the nests built in denser and higher vegetation resulted to have higher hatching success. The habitat surrounding the nest in terms of vegetation is frequently reported in literature as a critical discriminant for reproductive success in colonial seabirds, for its role in predation avoidance and external temperature regulation (Yasué and Dearden, 2006; Yorio et al 1995). We suggest the same important role of nest vegetation cover for reproductive outcome also in the two species of this study. Additionally, we hypothesise that this relation in a scenario of global temperature rising, human niche extension and associated modified-landscape, would threaten the reproduction and success of these species by directly impacting the vegetation of the natural nesting sites.

2.5.4 Findings criticism and robustness

The lack of identification of the breeding gull individuals at each nest is a potential limitation of this experimental design, as we considered the nest as a sampling unit. Any differences in specific subjects, for sex, weight and age remain unknown and, therefore, limit the assessment of how such traits affect HR and behavioural responses. Also, basal HR is known to vary substantially among individuals of the same species (see Weimerskrich et al., 2002; 20.3% of basal HR variation among individual of Wandering albatrosses). It is difficult to ascertain to which extent a possible over-representation of one of the two individuals of the pair in the sample could have affected our results. With this regard we also have to account the limited size of our sample. Indeed, the reduced sample size even if statistically significant is not enough to show strong evidence of biological significance.

Our results can be considered a first starting point for further investigation and better comprehension of the topic.

The extraction software and the dummy egg itself sometimes failed in data collection reducing the sample size for the analysis, both during basal and stress periods. These gaps in the dataset are most often attributed to hight frequency surrounding noise. Indeed, the dummy egg is a contact-based device also frequently subjected to the egg-roll characteristic behaviour of incubating birds. The software is a new tool that still requires improvements which can be more easily obtained after experimental application in different scenarios to point out the weaknesses. We believe that the methodology of repeating the analysis during the replicate phase, producing a supplement set of data, successfully help to partially overcome these limitations, by increasing the available sample size.

The overall experiment, as desired, did not negatively affect the reproductive success of the colonies, except for the Audouin's experimental area. The cause of this reduction in hatching success, however, is more likely to be associated with the location of the experimental site and not the experiment itself. In fact, the experimental area of the Audouin's gull was the one more exposed due to proximity to the walkway used by tourists and researchers to reach the beaches and the closest to the beach. This circumstance could it also be the reason behind the higher basal HR in Audouin's gull that led to a non-significant variation from basal to stress conditions during the Period II of the replicates days (table 2; results).

2.5.5 The dummy egg methodology

The use of the dummy egg as sampling tool in this research confirmed the literature findings, as a minimal invasive technique (Arnold et al., 2011; Nimon et al, 1996). For what we observed, the gull's behaviour remains unaltered and unaffected due to the presence of the artificial egg. The audio tracks and the temperature were well recorded in all the deployed intervals and no mechanical failures occurred. The absence of external wires or device appendixes bring to our artificial egg model advantages of compactness, avoiding birds stumbling over it and or being trapped and facilitating the deploying procedures. Moreover, in the audio file, the heartbeat results clearly identifiable for manual inspection (Annex 8). While the hardware proved to be highly user-friendly, the software could be improved. The heart rate values extraction and their resolution could be further improved, as -albeit to a lesser extent - the exclusion of external noises, such as seagull's vocalization and wind for better recognition of the heart beats. In general, the few adjustments we suggest include a more developed automatization that would allow faster and easier data analysis and therefore, the possibility of sampling and build bigger datasets. Finally, the improvement of the battery time-life together with an on-off remote switch, could allow to sample more data without interruption and further reduce disturbance associated with retrieving and deploying the devices.

This would potentially turn the dummy egg device into to one of the most useful tools for further and deeper investigation on seabird research during reproductive season with minimal impact and potential bias.

2.6 Conclusion

We found that the yellow-legged gull and the Audouin's gull as other seabird species are influenced by the bare presence of humans being within the area of the reproductive colonies. We were able to observe both a behavioural and physiological response that included an altered heart rate of the incubating adults and temporarily leaving the nests unattended, consistent with a predation-risk reaction. The detrimental consequences of human disturbance in particular in seabird reproductive colonies is a controversial topic, and several studies even theorised that chronic disturbance during reproduction could lead to a selection of those more tolerant individuals that could continue to breed under stressed conditions. This research represents a starting point on the physiology knowledge in terms of heart rate regarding the Yellow-legged and the Audouin's gull. Moreover, it provides a first insight of the potential threat that human presence represents for reproduction success and survival of these species.

Based on the heart rate response, the impact of anthropogenic disturbance on the colonies of the two species was similar even though the two species show a different strategy in relation with humans in terms of exploiting behaviour. This means that assumptions about human influences on seabirds, even on species like yellow-legged gull - considered opportunistic and easily adaptable to human presence and modified landscape - have to be carefully assessed. Based on this study, we confidentially suggest that the assessment of anthropogenic impact on specific species of seabird should be performed through a multidisciplinary approach. We believe that complementing behavioural and observational data together with physiological analysis and reproductive output of studied breeding pairs could generate important information on how species and populations deal with disturbance and anticipate potential impacts on their fitness.

The finding that vegetation structure plays a similar role on reproductive success of both species, in terms of hatching success, and the further confirmation that temperature affects the behaviour, and the physiology of the nesting adults should be considered a warning sign in an environmental global change scenario. Apart from climate change, human activities that may directly alter the vegetation in nesting colonies are likely to affect the survival of nesting seabirds, chicks, and eggs. Therefore, on top of the direct effect we discussed, we suggest that when it comes to evaluate the anthropogenic impact on seagulls' colonies, a wider approach accounting for the habitat vegetation changes should be taken in consideration as indirect additional human threat to gulls' fitness and survival. Therefore, mitigation measurements such as delimiting the nesting colonies and building predefined walking path through the colony's sites, conducting awareness campaigns during the reproductive season and constrain the duration of, and space out in time, the touristic activities in the areas could reduce gulls' stress and habitat degradation.

Further studies on this topic are essential to better understand the biological significance and the mechanism of anthropogenic presence and activities influence on these species. This would provide information for management plans and mitigating tools for the conservation of the yellow-legged gull and Audouin's gull and other species which inhabit similar habitats during reproduction, as to protect the coastal ecosystem. Ecosystem balance is complex articulate structure that needs a deep understanding if we aim to preserve and manage it, while intensify the threat derived from the continuous growth of human population.

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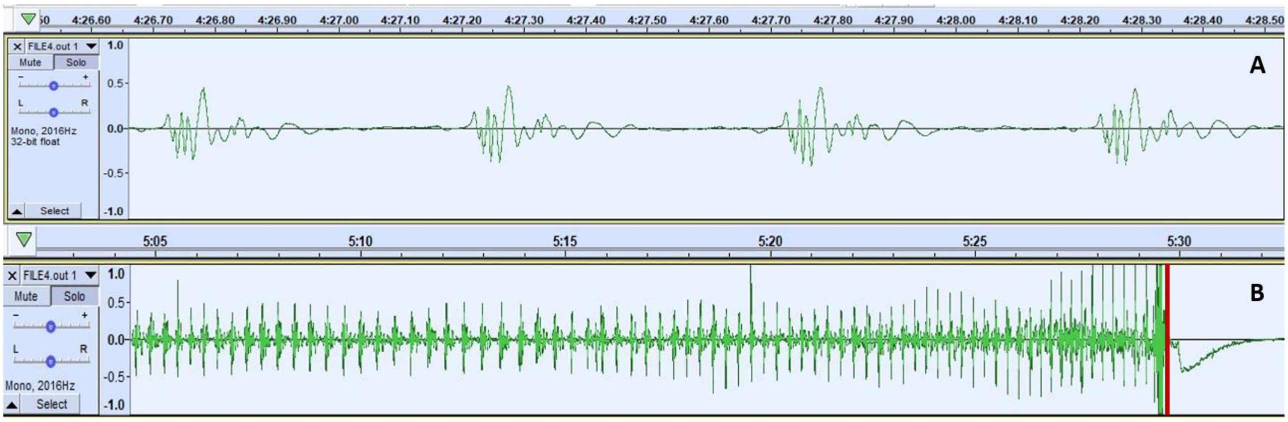
3. Annexes

Annex 1 *Nest set up during the experimental phase, to assess the effects anthropogenic disturbance on yellow-legged gulls and Audouin's gulls during the breeding season in 2021 on Deserta island, south coast of Portugal. Elements A and B represent respectively the dummy egg and the real egg in the nest. Element C stands for the thermohygrometer datalogger, and D for the wooded pole that identifies the nest.*

Annex 2 *Experimental area of Audouin's gull breeding colony during the experimental phase to assess the effects of anthropogenic disturbance on breeding individuals, conducted in 2021 on Deserta Island, south coast of Portugal. In the foreground on the left side, the video recording cameras (A) used for nest monitoring, various wooden pole to identify the experimental nests presence (B), and is recognizable a thermohydrometer (C).*



Annex 3 *Example of an interval record of a heartbeat track of yellow-legged gull opened in Audacity software. The shown soundtracks are normalized and amplified. Panel A shows heart beats during undisturbed conditions (Basal HR) and panel B shows heart beats during disturbance, right before the gull took-off - red vertical line (Stress HR) – N.B. the reported time scale of the two panels is not equivalent. Data acquired during the experiment assessing the anthropogenic disturbance effects on seabird colonies, during the reproductive season 2021 in Deserta Island, south coast of Portugal*

Annex 4 *Scores obtained by principal component analysis (PCA) to characterise the vegetation features of nests in both experimental and control areas for the Yellowed-legged gull and the Audouin's gull, during the experiment to assess anthropogenic disturbance effects on colonial seabirds, during the reproductive season 2021 in Deserta Island, south coast of Portugal. The variables included in the analyses were: percentage of vegetation coverage in the whole Nest, Cup, North, East, South, West, and vegetation maximum height in centimetres (Max Height- for more details please see Materials and Methods). The PCA extracted one component (PC1) with eigenvalue >1, which explained 59.31% of the variation. For variables transformations see Data Analysis in the Materials and Method section.*

		Principal component analysis			
	Variables	PC1	PC2	PC3	PC4
Eigenvalues		4.152	0.773	0.705	0.654
Standard Deviation		2.038	0.879	0.840	0.809
Proportion of Variance		0.593	0.110	0.101	0.093
Cumulative Proportion		0.593	0.704	0.804	0.898
Rotation Matrix (Loading vector)	Nest %	-0.476	0.062	0.016	-0.249
	Cup %	-0.341	-0.236	-0.061	0.770
	Max Hight	-0.416	-0.028	0.025	0.193
	North %	-0.351	-0.399	-0.389	-0.525
	East %	-0.365	-0.434	0.491	-0.077
	South %	-0.329	0.622	0.501	-0.132
	West %	-0.346	0.452	-0.593	0.092

Annex 5 *Egg dummy temperature variation, thermohygrometer nest microhabitat records and incubating gulls' absence period duration during experimental disturbance Mean ±SD, in yellow-legged gull (L. michahellis) and Audouin's gull (I. audouinii), in the first (Original) and second disturbance phase (Replicate) and in each of the two daily transect of walking-operator's disturbance event (Transect I:10:00 -10:30 h, Period I; Transect II: 14:00-14:30 h, Period II). Specifically: DE temperature variation_ gull's absence is the is the difference between the minimum and the maximum temperature of the dummy egg during the gulls' absence from incubation; DE temperature variation _10 min transect, is the difference between the minimum and the maximum temperature of the dummy egg during the 10 minutes of transect; TH temperature _ gulls' absence, is the nest microhabitat temperature during the gull's absence; Resume time, is the time taken to recover incubation duties after disturbance. Original and replicates refers to two difference phases of data collection obtained from the same nest at three-day intervals.*

		Larus michahellis				***Ichthyaetus audouinii***			
		Transect I		*Transect II*		*Transect I*		*Transect II*	
		Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N
Original	DE temperature variation_ gull's absence (°C)	2.15 ± 1.46	21	2.39 ± 2.04	20	4.47 ± 2.28	17	4.03 ± 1.79	18
	DE temperature variation _10 min transect (°C)	3.18 ± 1.98	21	3.2 ± 2.14	20	3.56± 2.30	22	3.27 ± 1.73	22
	TH temperature _ gulls' absence (°C)	29.91 ± 3.71	19	28.48 ± 5.00	18	38.47 ± 5.41	18	36.12 ± 3.46	19
	Resume Time (Min)	4.36 ± 2.21	20	4.83 ± 2.651	19	2.88 ± 2.39	18	2.09 ± 1.63	19
Replicate	DE temperature variation_ gull's absence (°C)	3.18 ± 1.45	20	2.37 ± 1.94	18	3.57 ± 2.298	18	3.97 ± 1.79	16
	DE temperature variation _10 min transect (°C)	3.31 ± 1.77	22	3.19 ± 1.93	22	3.34 ± 2.08	20	3.24 ± 1.71	20
	TH temperature _ gulls' absence (°C)	33.21 ±3.70	20	28.65 ± 2.83	18	34.23 ±5.46	18	36.07 ± 3.42	16
	Resume Time (Min)	3.58 ±2.21	20	4.89 ± 2.70	18	3.24 ± 2.40	18	2.06 ± 1.62	16

Annex 6 *Generalised linear mixed models (GLMM) on the variation of the basal heart rate (Basal HR) in yellow-legged gull (YLG) and Audouin's gull (AG). Explanatory variables include gull species, microhabitat temperature (TH temperature, °C) time of day that is divided into three distinct periods in which walking-operator's disturbance was performed: Period I, which began with the deployment of the dummy egg (9:00-9:30 h) and finished 30 minutes after the end of the Transect 1 (around 10:00 -10.30 h; Reference category); Period II, that began at the end of Period I and ended 30 minutes after the end of the Transect 2 (around 14:00 - 14.30 h); and period III, that start at the end of Period II and ended with the retrieval of the dummy eggs (around 16:30/17; see data extraction paragraph in material and methods). The number in bold identifies a significant effect.*

		Basal HR			
		estimates	t	p	Main effects
TH Temperature		-0.937	-2.058	**0.040**	The higher the temperature the lower the heart rate
Species		-13.527	-1.772	0.076	_
Time of day	Period II	-5.604	-1.889	0.059	The lowest Basal HR is in Period III
	Period III	-6.413	-2.141	**0.032**	

Annex 7 *Model selection results for candidate models describing the hatching success in the yellow-legged gull and Audouin's gull. AIC's is the Akaike's Information Criterion values of each model. PC1 - principal component extracted from the Principal Component Analysis on the nest vegetation characterization data; DE Temperature - difference between the minimum and the maximum temperature during the gull absence of the nest, recorded by the dummy egg; Resume Time - the time in minutes between the gull's take off from the nest and incubation recovery.*

Candidate model (explanatory variables)	AIC	Difference
PC1 + DE Temperature	1476.5	0.5
PC1 + DE Temperature + Resume Time	1477	3.4
PC1 + DE Temperature + species	1480.4	0.4
PC1 + DE Temperature + Resume Time + species	1480.8	-

Annex 8 *Mean (±SD) of Basal HR in each of the three periods of the day and the Resume Time in the two different transect operated in each experimental day, during Original and Replicates, for yellow-legged gull (Larus michahellis) and Audouin's gull (Ichthyaetus audouinii). Original and Replicate refers to two difference phases of data collection obtained from the same nest at three-day intervals. Period I, began with the deployment of the dummy egg (9:00-9:30 h) and finished 30 minutes after the end of the Transect I (around 10:00 -10.30 h; Reference category); Period II, began at the end of Period I and ended 30 minutes after the end of the Transect II (around 14:00 - 14.30 h); and period III, start at the end of Period II and ended with the retrieval of the dummy eggs (around 16:30/17; see data extraction paragraph in material and methods). Transect I and II corresponds to the two walking-operator's disturbance events of every experimental day. The number in bold identifies a significant difference.*

		Original vs Replicate							
		Larus michahellis				*Ichthyaetus audouinii*			
		Original		*Replicate*		*Original*		*Replicate*	
Variable	Interval	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N	Mean ± SD	N
Basal HR	Period I	151 ± 40	18	135 ± 34	18	158 ± 20	18	147 ± 23	18
	Period II	**148 ±25**	**18**	**126 ± 18**	**18**	140 ± 17	18	142 ± 18	18
	Period III	147 ± 34	18	139 ± 24	18	149 ± 28	18	134 ± 21	18
Resume time	Transect I	4.30 ± 2.60	20	4.40 ± 1.68	20	3.11 ± 1.99	18	2.56 ± 1.34	18
	Transect II	5.26 ±3.16	19	4.44 ± 1.95	18	2.05 ± 1.93	19	2.06 ± 1.14	16